The Dpp Morphogen Gradient Regulates Growth and Patterning in the *Drosophila* Wing Imaginal Disc

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«Wisdom comes from experience. Experience is often a result of lack of wisdom.»

Terry Pratchett

To my family, specially to my girls.

Contents

Summary

Morphogens are small molecules that diffuse from a defined source and thereby establish a concentration gradient in tissues that regulates growth and patterning. The signaling pathways initiated by morphogens are important in the development of all metazoans. It is therefore no surprise that these pathways and their functions are highly conserved from flies to humans. One of the most studied morphogens is Dpp, the Drosophila homolog of BMP. In *Drosophila*, Dpp regulates the anteroposterior patterning and growth of tissues, such as the prospective wing. In the wing disc, it is produced at the anteroposterior boundary and spreads to generate a gradient that regulates tissue growth and patterning of the disc.

Dpp exerts its effect by binding to the receptors Tkv and Put. This triggers a signaling cascade that culminates in the expression of downstream genes. Depending on the concentration of Dpp, the strength of the signal varies and different set of genes will be expressed.

In addition to tissue patterning, Dpp also regulates growth. Both processes depend on the repression of the transcription factor Brk. In contrast to patterning, where the morphogen gradient is translated into the differential expression of target genes, the rates of division in all regions of the developing tissue are the same. The mechanism by which a graded signal is translated into a uniform response remains obscure.

In this thesis, I studied the dual role of Dpp in development by analyzing how the gradient is established and how it regulates growth. I present evidence that the diffusion of Dpp throughout the tissue is slow, and the gradient that Dpp creates is stable over time. This hindered diffusion allows for the formation of a stable steady state gradient that regulates the patterning of the wing.

In addition, I present evidence that the Dpp gradient is strictly required for growth. Without Dpp emanating from the anteroposterior boundary, cells from the prospective wing are unable to grow, and development is arrested. A graded signal, however, is not required for homogenous growth. Replacing the Dpp gradient with a uniform source of Dpp throughout the pouch leads to normal growth, although patterning is abolished. The role of Dpp in growth is then to signal cells at the medial region at a level high enough to repress Brk.

In summary, I show that a Dpp gradient is required for patterning but that growth is independent of this gradient. To promote growth the level of the morphogen needs to exceed a threshold such that Brk expression is abolished in the medial region of the developing wing.

Zusammenfassung

Morphogene sind kleine Moleküle, die von einer definierten Quelle ausgehend einen Konzentrationsgradienten im Gewebe etablieren, welcher sowohl Wachstum als auch Musterbildung des Gewebes reguliert. Die Signalwege, die durch Morphogene eingeleitet werden, sind entscheidend für die Entwicklung aller vielzelligen Lebewesen. Es ist daher nicht überraschend, dass diese Signalwege und ihre Funktionen von der Fliege bis zum Menschen hochkonserviert sind. Eines der am meisten untersuchten Morphogene ist Dpp, das *Drosophila* homolog von BMP. In *Drosophila* ist Dpp die anteroposterior Musterbildung und das Wachstum von Gewebe, wie z.B. künftige Flügel, veratnwortlich. In der Flügelscheibe wird es an der anteroposterioren Grenze produziert verbreitet sich von dort aus, wodurch ein Gradient generiert wird, der das Gewebewachstum und die Musterbildung der Scheibe reguliert.

Dpp wirkt, indem es an die Rezeptoren Tkv and Put bindet. Dies leitet eine Signalkaskade ein, die in der Expression von nachgeschalteten Genen ihren Höhepunkt findet. Abhängig von der Konzentration von Dpp variiert die Stärke des Signales, und eine unterschiedliche Kombination von Genen wird exprimiert.

Zusätzlich zu der Musterbildung des Gewebes reguliert Dpp auch dessen Wachstum. Beide Prozesse sind abhängig von der Unterdrückung des Transkriptionsfactors Brk. Die Funktion des Morphogengradienten während der Musterbildung resultiert in der Expression von verschiedenen Genen, abhängig von der Position im Gewebe und der Intesität des Dpp Gradienten. Während dem Wachstum hingegen teilen sich die Zellen in allen Regionen des Gewebes mit der gleichen Rate, unabhängig von der Stärke des Dpp signals. Der Mechansimus, der zur Übersetzung eines Gradient in eine uniforme Wachstumsrate führt, verbleibt unklar.

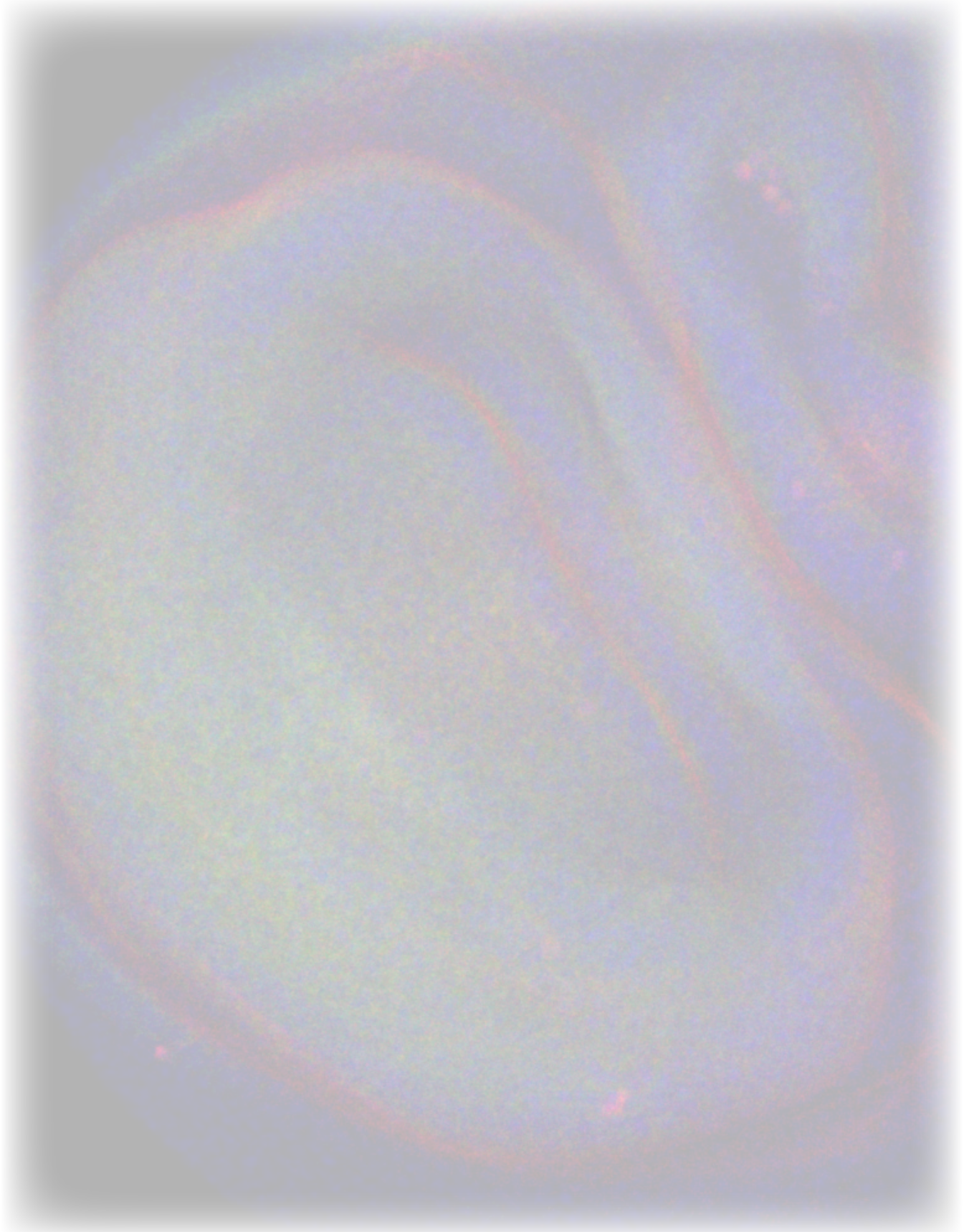
In dieser Arbeit untersuchte ich die duale Rolle von Dpp während der Entwicklung und Wachstum. Dafür analysierte ich die Enstehung des Gradienten und liefere Beweise, dass Dpp langsam durch das Gewebe diffundiert und dass der Dpp-Gradient im Laufe der Zeit stabil bleibt. Die verlangsamte Diffusion erlaubt die Bildung eines anhaltend stabilen Gradienten, der so die Musterbildung des Flügels reguliert.

Zusätzlich zeige ich, dass der Dpp-Gradient nur bedingt für Wachstum

benötigt wird. Ohne das Ausstrahlen von Dpp von der anterioposterioren Grenze aus, ist es für die Zellen des künftigen Flügels nicht möglich zu wachsen und die Entwicklung kommt zum Stillstand. Allerdings wird für das homologe Wachstum kein fein abgestufter Gradient benötigt, sondern nur eine hohe Konzentration in der medialen Region des Flügels. Denn das Austauschen des Gradienten mit einer uniform über die ganze Fluegeltasche verteilte Quelle von Dpp führt zwar zu normalem Wachstum, allerdings auf Kosten der normalen Musterbildung eines Flügels. Während dem Wachstum eines Gewebes ist die einzige Rolle von Dpp, in den medialen Regionen, wo die Konzentration am höchsten ist, die Expression von Brk zu unterdrücken.

Zusammenfassend kann ich beweisen, dass ein Dpp-Gradient für die Musterbildung, aber nicht für das Wachstum benötigt wird. Für das Wachstum muss die Konzentration an Dpp nur eine Schwelle überschreiten, die in der medialen Region des Flügels die Expression von Brk verhindert wird.

Chapter 1. General introduction



Morphogens

How a single cell gives rise to a multi-cellular adult organism is a complex and fascinating process termed development. During development, cells divide, migrate and group according to their fate, growth and patterning are coordinated to create the different tissues, organs and appendages. Several signaling pathways are involved in regulating the body development, and they need to be controlled both spatially and temporally. The activation and synchronization of the signaling pathways is possible thanks to a group of molecules named morphogens. The pathways triggered by morphogens regulate growth and provide each cell with positional information.

Morphogens are small signaling molecules that are typically produced from a defined group of cells and diffuse through the tissue to generate a concentration gradient (Rogers and Schier, 2011). When they bind to their receptors they activate signaling cascades that ultimately regulates the expression of genes involved in the patterning of developing tissues. The concentration gradient of the morphogen is key for patterning: at different signal thresholds the expression of different subsets of genes is activated. This idea was first proposed by Lewis Wolpert through the model known as the French Flag (Wolpert 1969). After almost 50 years of research, all the gathered evidence has proven this model correct.

Several pathways are regulated by secreted morphogens, including TGF-β, Wg/Wnt and Hh. These morphogens and their signaling pathways are highly conserved, retaining analogous functions from flies to humans. They regulate many processes, from the early axis formation in embryos (Driever and Nüsslein-Volhard, 1988; Irish and Gelbart, 1987) to tissue homeostasis in adults (Radtke and Clevers, 2005). Given these critical functions, it is not surprising that defects in morphogenetic signaling pathways cause severe malformations during development and have been linked to many disorders and cancer in adults (Davis et al., 2016; Fogarty et al., 2005; Reya and Clevers, 2005).

The Dpp morphogen gradient

Decapentaplegic (Dpp) is one of the most studied morphogens as it is

involved in the development of many tissues across species. It is a member of the TGF-β superfamily and is the homolog of the vertebrate BMP2/4 (Matsuda et al., 2016). Dpp is a key player during the development of *Drosophila melanogaster* larvae, it determines the anteroposterior patterning of several of the larval imaginal discs (Held, 2005). Imaginal discs are pockets of epithelial cells that grow in the developing larva and differentiate during the pupal stage to give rise to adult appendages. The development of the imaginal discs is orchestrated by the combined action of various morphogens, which via their signaling gradient establish compartments that will afterwards develop into the different structures composing the appendages (Held, 2005). The wing disc is the largest of the imaginal discs and the most studied one. It grows from around 50 cells at the beginning of the larval period to 50.000 cells at pupariation, the disc then morphs into the adult wing, hinge and notum (García-Bellido and Merriam, 1971). The role of morphogens in the development of the wing disc has been extensively studied. During the larval development, three morphogens coordinate to pattern the wing disc: Hh, Wg and Dpp (Nellen et al., 1996). Together, they determine two spatial axes, which constitute the boundaries of the four developmental compartments: dorsal, ventral, anterior and posterior. Wg, expressed at the dorsoventral boundary, produces a gradient that regulates the dorsoventral patterning (Zecca et al., 1996). Hh, regulated by Engrailed, is produced at the posterior compartment, and diffuses towards the anterior compartment. A stripe of cells from the anterior compartment, close to the boundary receive Hh signaling, and as a consequence produce Dpp (Basler and Struhl, 1994). Secreted Dpp diffuses to create a gradient that coordinates the anterior and posterior patterning (Fig. 1A) (Zecca et al., 1995).

The Dpp morphogenetic gradient regulates the expression of several genes during larval development. Dpp binds to its receptors Thickveins (Tkv) and Punt (Put). Tkv and Put are Type I and II serine/threonine kinase receptors, respectively. When bound to Dpp, Put drives the phosphorylation-mediated activation of Tkv, and active Tkv phosphorylates Mothers against Dpp (pMad) (Derynck, 1994). Activated pMad binds to Medea and migrates to the nucleus, where together regulate the expression of several genes, e.g. Spalt major (Salm), Optomotor blind (Omb) or Brinker (Brk) (Fig. 1B) (Affolter and Basler, 2007).

The Dpp pathway is critical for growth and patterning: the absence of

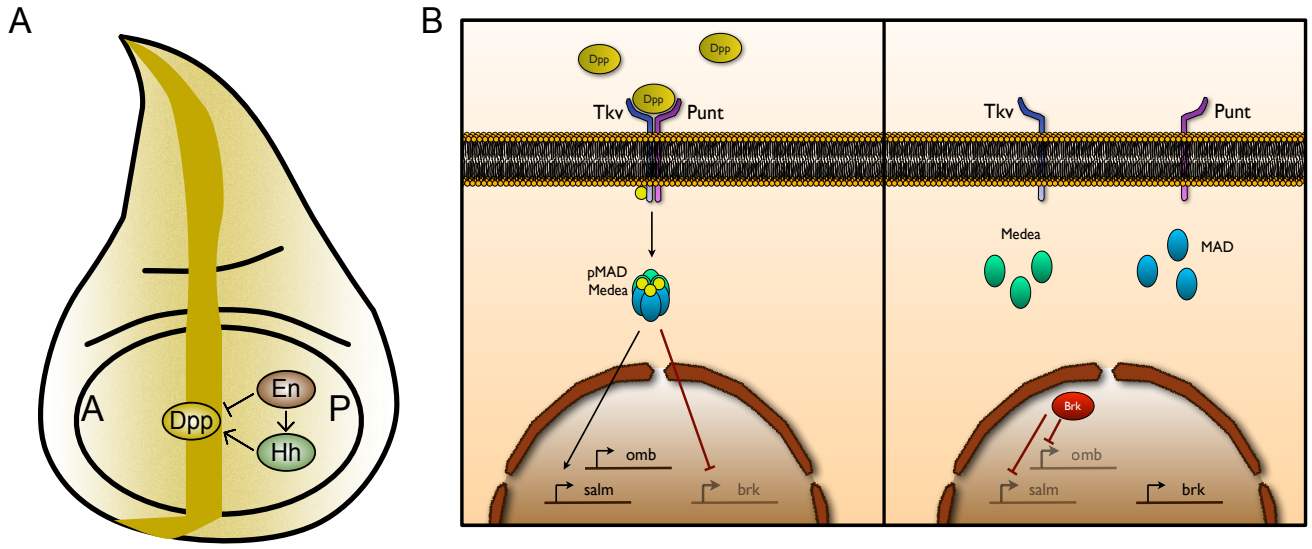


Figure 1. The Dpp pathway

A) Dpp expression is repressed by Engrailed and activated by Hedgehog. Dpp is produced from a stripe of cells at the anteroposterior boundary. B) Secreted Dpp binds to the receptors Tkv and Punt to phosphorylate Mad and regulate the expression of target genes. In the absence of Dpp signaling, Brk is expressed and represses Dpp target genes.

Dpp results in growth arrest in several imaginal discs and defects in the pattern of adult structures (Restrepo et al., 2014; Singer et al., 1997). Both growth and patterning need to be coordinated to drive cell fate and ensure the proper formation of the adult structures (Schwank and Basler, 2010). The patterning of the disc is regulated by differential gene expression profiles elicited by the morphogenetic gradient. In contrast, despite the graded Dpp signal growth is homogeneous throughout the imaginal disc. How the two processes are coordinated and how a graded signal translates to homogenous growth are open questions.

Dpp and growth

Shortly after the role of Dpp in regulating patterning of the wing imaginal disc was found, researchers realized that it was also required for disc growth. Analysis of dpp LOF alleles in the wing discs revealed severe growth defects (Burke and Basler, 1996; Schwank et al., 2008; Teleman and Cohen, 2000). Ectopic expression of Dpp, on the other hand, leads to disc overgrowth (Schwank et al., 2011; Wartlick et al., 2011). The same effect is observed if the Dpp receptors are manipulated. Mutants for the Dpp type I receptor Tkv are unable to grow, while the expression of a constitutively active form of Tkv, Tkv^{QD}, leads to overproliferation in the prospective

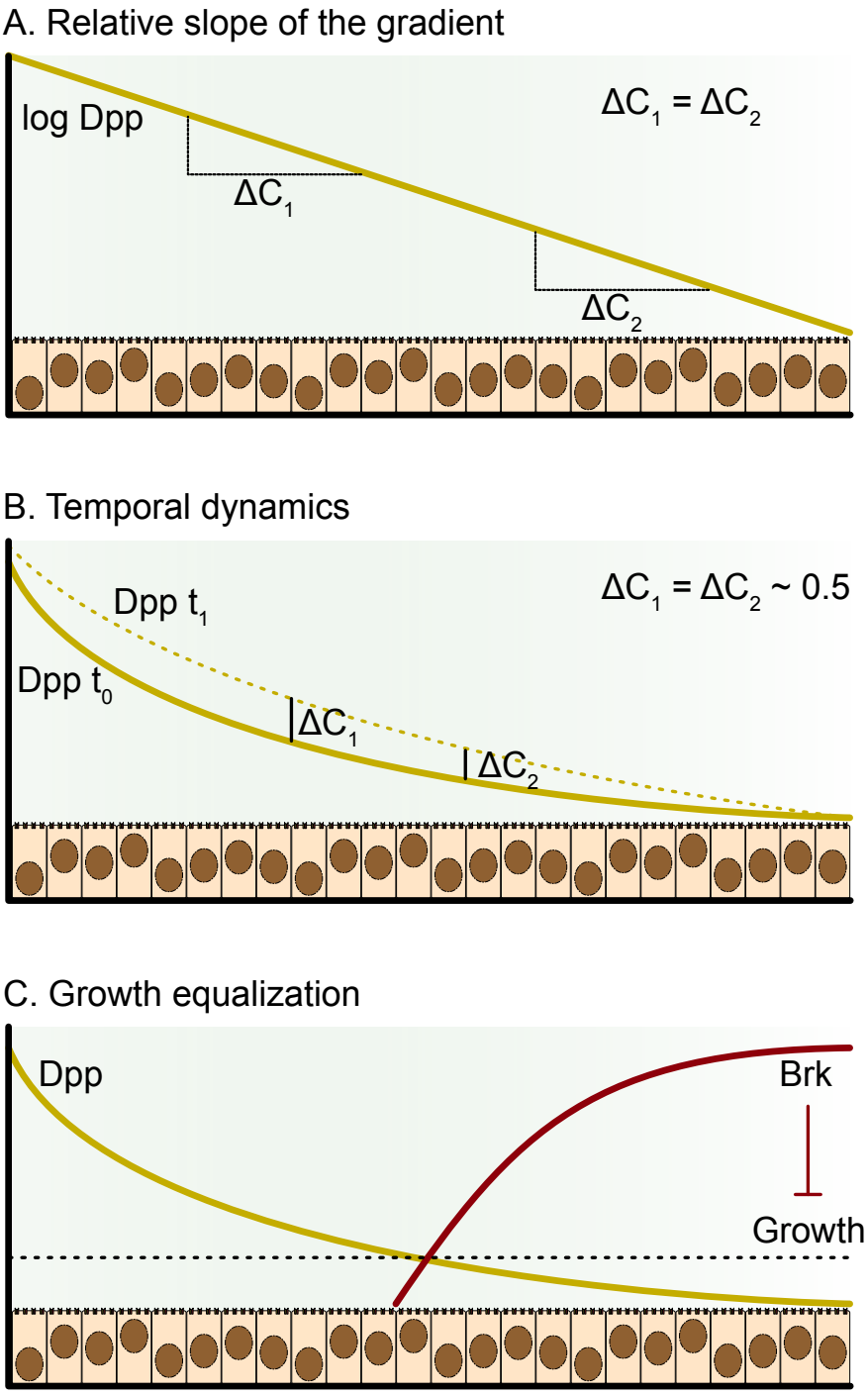


Figure 2. Growth regulation by Dpp

A) In the relative slope, all cells sense the same relative decrease on Dpp concentration (ΔC) regardless of their position.
B) In temporal dynamics, cells divide when the concentration of Dpp increases a 50%. Dpp accumulates equally in every region of the disc (ΔC), and when the accumulation of the morphogen reaches a threshold of around a 50% increase, cells divide.
C) Growth equalization model. Lateral cells have an inherent faster division rate. Brk slows down growth of lateral cells, and Dpp impedes Brk expression in medial cells to equalize growth throughout the disc.

wing (Burke & Basler 1996). Several research groups have invested years in trying to explaining how a graded signal can lead to homogenous growth, and various models have been proposed to explain this conundrum (Fig. 2).

Day and Lawrence proposed that cells do not grow according to the amount of morphogen they receive, but to the relative slope of the gradient (Day and Lawrence, 2000), i.e. the higher the local differences in Dpp, the faster cells divide. This morphogen-slope model (Fig. 2A) would resolve the issue of the homogeneous growth vs. graded signal of Dpp, as

the local differences in the gradient would be the same in any given region of the disc. This model also solves the growth arrest that occurs at the end of development, as cells will stop dividing once the disc grows enough and the morphogen slope flattens. However, later findings showing that a uniform Dpp signal could still drive growth made this model obsolete (Schwank et al., 2011; Wartlick et al., 2011).

Could cells sense the increase in Dpp instead of the slope? That is what Wartlick and colleagues proposed with the temporal dynamics model, schematized in Fig. 2B (Wartlick et al., 2011, 2014). According to this model, cells detect changes in the Dpp concentration while the morphogen accumulates. Once cells detect a relative constant increase of Dpp (of around 50%), they will divide. Since the relative increase in Dpp is equal in the prospective wing, growth will be homogeneous despite being regulated by a graded signal. This model also explains the growth arrest at the end of development. The bigger the tissue grows, the harder it will be to achieve the required increase in Dpp concentration, thus reducing proliferation. After a certain threshold, growth will stop.

Although very elegant at first sight, there are some pitfalls to the temporal dynamics model. When observing whole disc dpp and brk double mutants, one can see that the overgrowth is not even throughout the disc. Lateral regions of the disc overgrow, while medial cells retain normal growth. These double dpp and brk mutant discs phenocopy a brk LOF, which shows the same lateral overgrowth (Schwank et al., 2008, 2011). In addition, clones lacking Dpp signaling and Brk overgrow when they are located at the lateral regions of the disc, while they retain normal growth when they are in the medial region (Schwank et al. 2012). Two conclusions can be extracted from these data. First, disc cells without Dpp signaling can grow as long as Brk is missing, suggesting that they do not require changes in Dpp concentration to divide. Second, lateral cells tend to grow faster than medial cells, and Brk is required to repress lateral growth. Supporting this observation, it was found that Brk inhibits growth partially via the repression of Myc (Doumpas et al., 2013). To explain this dilemma, our group postulated the growth equalization model (Fig. 2C). According to this model, Dpp is required by medial cells to repress Brk and equalize inherent growth differences in the wing disc (Schwank et al., 2008). There are two main reasons for the restricted growth of medial cells. First, it was proposed that the mechanical forces affecting growing tissues would exert

pressure over medial cells, compressing them and hindering proliferation (Aegerter-Wilmsen et al., 2007). However, this idea remains theoretical, with no experimental evidence. Second, it is possible that another pathway, independent of Dpp, inhibits growth in the medial region. Supporting that idea, it was found that the Fat signaling pathway represses growth of medial cells (Schwank et al., 2011).

Recently, the role of the Dpp gradient in growth has been challenged. Akiyama and collaborators showed that discs where the Dpp gradient was completely abolished were able to grow almost normally. By cleaving dpp from the A/P boundary, patterning was impaired but disc cells were able to grow normally. This suggested that the Dpp emanating from the A/P boundary is not required for growth during later stages of larval development, but only for patterning (Akiyama and Gibson, 2015). These authors propose that, as long as a source for Dpp exists during early development or from a source other than the central stripe, discs are able to grow. It was also shown that if Dpp is trapped at the A/P boundary, abolishing the gradient formation completely, discs are able to grow partially. Lateral cells grew in absence of a normal Dpp gradient, but medial cells were unable to grow properly when Dpp diffusion was abolished (Harmansa et al., 2015). Therefore, it seems that lateral cells do not require a gradient, but medial cells need Dpp diffusion to grow normally, consistent with the growth equalization model. It remained to be tested, whether medial cells require a morphogen gradient or they just need to be exposed to Dpp during development to repress Brk. Answering this question was one of the goals of my thesis.

Gradient formation and scaling

Over the course of three days, the wing disc grows from 50 cells to around 50.000. During this time, the Dpp gradient adapts, extending and scaling to stably regulate gene expression in the expanding tissue. The mechanism by which Dpp diffuses through the disc and establishes a steady state gradient has been extensively studied but a consensus is missing.

Dpp morphogen dispersal can be explained through five different (and not mutually exclusive) hypotheses: 1) Dpp diffuses freely, both the gradient slope and length are regulated by morphogen uptake (Zhou et al., 2012); 2)

The diffusion of Dpp is slow, hindered by transient binding to extracellular components (Schwank et al., 2011b); 3) There is a facilitated diffusion, where molecules secreted from the lateral region of the disc reduce the hindering effect to which Dpp is submitted (Vuilleumier et al., 2010); 4) Dpp is captured and dispersed by the receptors located on long cytoplasmic protrusions called cytonemes, extending from lateral cells towards the source (Hsiung et al., 2005); or 5) Dpp is transported via transcytosis, i.e. successive rounds of endo- and exocytosis between neighboring cells (Entchev et al., 2000). A more comprehensive review of the field is presented in chapter 4 or in the reviews by Restrepo et al. 2014 and Müller et al. 2013.

Regardless of the mechanism by which Dpp diffuses, there are various extracellular components involved in fine-tuning the signaling gradient. The most prominent ones are the glypicans. Glypicans are a family of heparan sulphate proteoglycans (Filmus and Selleck, 2001; Sarrazin et al., 2011). In mammals, six members of this family of molecules have been identified, while in Drosophila, the family is composed by Division abnormally delayed (Dally) and Dally-like protein (Dlp). Dally and Dlp are expressed during development and form an extracellular chain of glycosaminoglycans that acts as a binding site for various molecules (Filmus and Selleck, 2001). This lattice has been found to bind Dpp, impacting Dpp signal transduction and the signaling gradient (Akiyama et al., 2008; Jackson et al., 1997). Because of this sequestering effect, it has been proposed that glypicans play a key role in the gradient formation, either by hindering Dpp or permanently binding it, reducing the amount of free diffusible morphogen (Akiyama et al., 2008; Belenkaya et al., 2004; Zhou et al., 2012).

If glypicans are in charge of fine-tuning Dpp signaling and diffusion, their levels need to be adjusted to the requirements of the pathway. A secreted molecule, Pentagone (Pent), was found to negatively regulate Dpp signaling through the interaction with glypicans (Vuilleumier et al., 2010). More specifically, high levels of Pent reduce BMP signal transduction but increase the gradient width (Hamaratoglu et al., 2011; Vuilleumier et al., 2010). Pent is repressed by Dpp signaling, therefore its expression domain is restricted to the lateral regions of the disc. Pent is secreted and diffuses towards the medial region of the disc, where it interacts with glypicans, triggering their internalization and degradation. The degradation of glypicans would translate into a lower amount of Dpp bound to the cell surface, which in turn reduces the amount of Dpp signaling and potentially enhan-

ces Dpp spreading (Norman et al., 2016).

In order to fully understand how the Dpp gradient is established and its role in growth regulation, it is important to dissect the precise function of glypicans and Pent in Dpp diffusion, the establishment of the gradient steady state, and signal transduction. By determining the role of these molecules during larval development, we may finally understand how the Dpp gradient is regulated, and how the morphogen levels regulate growth.

Goals

The aim of my project was to find the mechanisms by which Dpp regulates growth and patterning. To achieve that goal, I devised novel transgenic flies to study how the tissue is able to coordinate the regulation of the morphogenetic gradient and link it to homogenous growth.

I generated transgenic dpp constructs expressing a tagged Dpp to study the effect of Dpp LOF in various scenarios and the diffusion of Dpp throughout the imaginal disc. I have found that Dpp growth requires the Dpp that emanates from the A/P boundary, although a graded signal is not critical for growth. Moreover, growth can still be promoted via a homogenous signal of Dpp, although the gradient is required for the patterning of the tissue. This work is discussed in Chapter 3 and has led to a publication in the journal Elife (Sanchez Bosch et al., 2017).

In Chapter 4, I present a novel approach to study the Dpp gradient. By modifying the endogenous *dpp* locus, I have observed for the first time the endogenous Dpp gradient. I have measured the Dpp degradation rate and confirmed that extracellular Dpp remains for long periods of time trapped around the cells. My data points to a hindered diffusion of the morphogen.

In this dissertation, I also present a new method for the genetic toolbox of Drosophila: in Chapter 3, I describe the adaptation of the Anchor Away to fly, a convenient tool to induce protein LOF (Haruki et al., 2008). I have proved the efficiency of the technique by generating LOF phenotypes of Brk and Pygopus and assayed the technique in comparison to RNAi.

In summary, my results have elucidated new mechanisms to keep homogeneous growth under the control of a morphogenetic gradient. The

tools I have generated allowed us to study how Dpp diffuses and how the gradient is regulated by cells of the wing imaginal disc.

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Chapter 2: Anchor-away - a fast, reliable and reversible technique to inhibit proteins in *Drosophila melanogaster*

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Abstract

Several techniques have been developed to study specific gene function in loss of function situations. In *Drosophila*, RNAi and the generation of mutant clones are widely used. However, both techniques have the limitation that there is a significant lag time before gene function is abolished. Given the relatively quick development of *Drosophila*, this is a serious impediment to study gene function. Here we describe the adaptation of the anchor-away technique for use in *Drosophila*. Anchor-away was developed in yeast to quickly and efficiently abrogate the function of nuclear proteins by sequestering - anchoring - them away in a different compartment. The required components are present in the cells, and system is triggered by the addition of rapamycin, resulting in a very rapid generation of a loss of function situation. We provide the proof of principle for the system by producing loss of function situations for two nuclear proteins – Pygopus and Brinker. The system allows us to study the effects of the loss of function of any protein during any time window, and at the same time circumvents difficulties, such as off-targets or variable phenotypes, which are inherent in for example RNAi.

Introduction

Loss-of-function (LOF) experiments have been performed for decades to study gene function. In *Drosophila*, several methods have been developed and extensively used. Mutagenesis screens have led to the discovery of the function of hundreds of proteins and were integral in the quest for identifying pathway components (Nüsslein-Volhard & Wieschaus 1980; St Johnston 2002; Jenny & Basler 2014). To facilitate the study of the function of a specific gene, other techniques were developed. Of these techniques, P-element insertions (Cooley et al. 1988) and RNAi downregulation (Fire et al. 1998) were the most widely adopted and extensively used in flies. One reason for this was the generation of libraries where almost every gene in the genome was targeted. Combined with other tricks in the genetic toolkit of *Drosophila*, one could control when and where a certain mutant was produced and thereby also study genes that otherwise were lethal. The preeminent approach that allowed the generation of specific LOF in cells and tissues was the generation of mitotic recombinant clones (Xu & Rubin

1993). Later the combination of inducible or stage specific drivers with an RNAi construct to down-regulate the expression of a gene of interest rose to equal prominence (Dietzl et al. 2007). These methods key weapons in the arsenal of scientists performing reverse genetics Although powerful, these methods have a major drawback: they do not directly target the protein but act on the gene or the mRNA and so are very sensitive to issues such as protein half-life and there is always a delay before the loss of function takes effect in the tissue (Boutros & Ahringer 2008).

To overcome this problem, several approaches have been developed to achieve a more rapid and efficient loss-of-function by targeting the protein directly. These methods rely on targeted protein degradation, cleavage or sequestering (Harmansa et al. 2017; Haruki et al. 2008; Caussinus et al. 2012). One of these methods, developed in yeast, is the anchor-away technique. The loss-of-function is achieved by sequestering the target protein in another compartment of the cell where it is unable to perform its physiological function. This sequestering is triggered by the addition of rapamycin, allowing investigators to trigger the LOF at any time point. The effect of the anchor-away method is essentially instantaneous as all the necessary components are already present in the cell, and the loss of function is triggered by the addition of rapamycin (Haruki et al. 2008).

The technique is based on a binary system whose components have to be integrated beforehand: an anchor protein (by which the protein of interest will be sequestered) and an engineered target protein. The anchoring process is based in the interaction between the human FK506 binding protein (FKBP12), and the 11 kD, FKBP12-rapamycin-binding (FRB) domain of the human mTor (Chen et al. 1995; Belshaw et al. 1996). Rapamycin binds to a FKBP12, and this creates an interaction surface for FRB, which binds and forms a tight ternary complex (Chen et al. 1995). By tagging the anchor with FKBP12 and the target with FRB, the two will bind strongly to each other after the addition of rapamycin. As a consequence, the target will be sequestered to the subcellular compartment where the anchor is located (Fig 1A).

There are various possibilities when anchoring proteins away, depending in the subcellular location of the protein of interest. For nuclear proteins, a cytoplasmic anchor is the obvious choice, and ribosomal proteins have been shown to be suitable for this, as once ribosomes are assembled, they will remain cytoplasmic (Haruki et al. 2008). In addition, ribosomal prote-

ins translocate to the nucleus after biosynthesis, where they combine with the different rRNA molecules to assemble ribosomes. Afterwards, the large and small ribosomal complexes are translocated to the cytoplasm (Zemp & Kutay 2007; Köhler & Hurt 2007). In that process, the target protein will also bind to the ribosomal protein anchor, and afterwards is translocated to the cytoplasm, where it is prevented from going back to the nucleus. If the target protein is cytoplasmic, a membrane-bound anchor has been shown to be efficient (Tsuchiya et al. 2013). The development of a suitable anchor is key and will depend entirely in the cellular localization of the target that one wants to anchor away.

In the present work, we adapted the anchor-away technique to *Drosophila* – we devised a ribosomal protein anchor to be able to study LOF of *Drosophila* nuclear proteins. As a proof of principle, we have tested the technique with two nuclear factors from independent pathways – Pygopus and Brinker. The LOF phenotypes confirmed the systems specificity and efficiency.

Results

Adapting the Anchor-Away system to Drosophila

Adapting the Anchor-Away (AA) to *Drosophila melanogaster* required the generation of an anchor appropriate for functional target. In addition, the modification of various genes was a required: To avoid toxicity, a rapamycin resistant we needed to confer rapamycin-resistance to *Drosophila*. To this end we introduced in flies carrying a $tor^{\Delta P}$ null allele a genomic rescue construct for Tor in which the CDS has the mutation S1956T. This mutation renders Tor rapamycin resistant (Zhang et al. 2000). To avoid potential complication, we also abolished the expression of an endogenous FK506 binding protein the *Drosophila* homolog of the yeast FPR1, FK506-bp2. For this crossed in the Kyoto stock 205044, which carries a P-element insertion in the second exon of FK506-bp2 (Toba et al. 1999), abolishing its expression.

Next, we generated the protein anchor, which has to be expressed ubiquitously and at high levels to ensure efficient sequestering of the target. We selected the ribosomal protein Rpl13a, the homologue of the protein

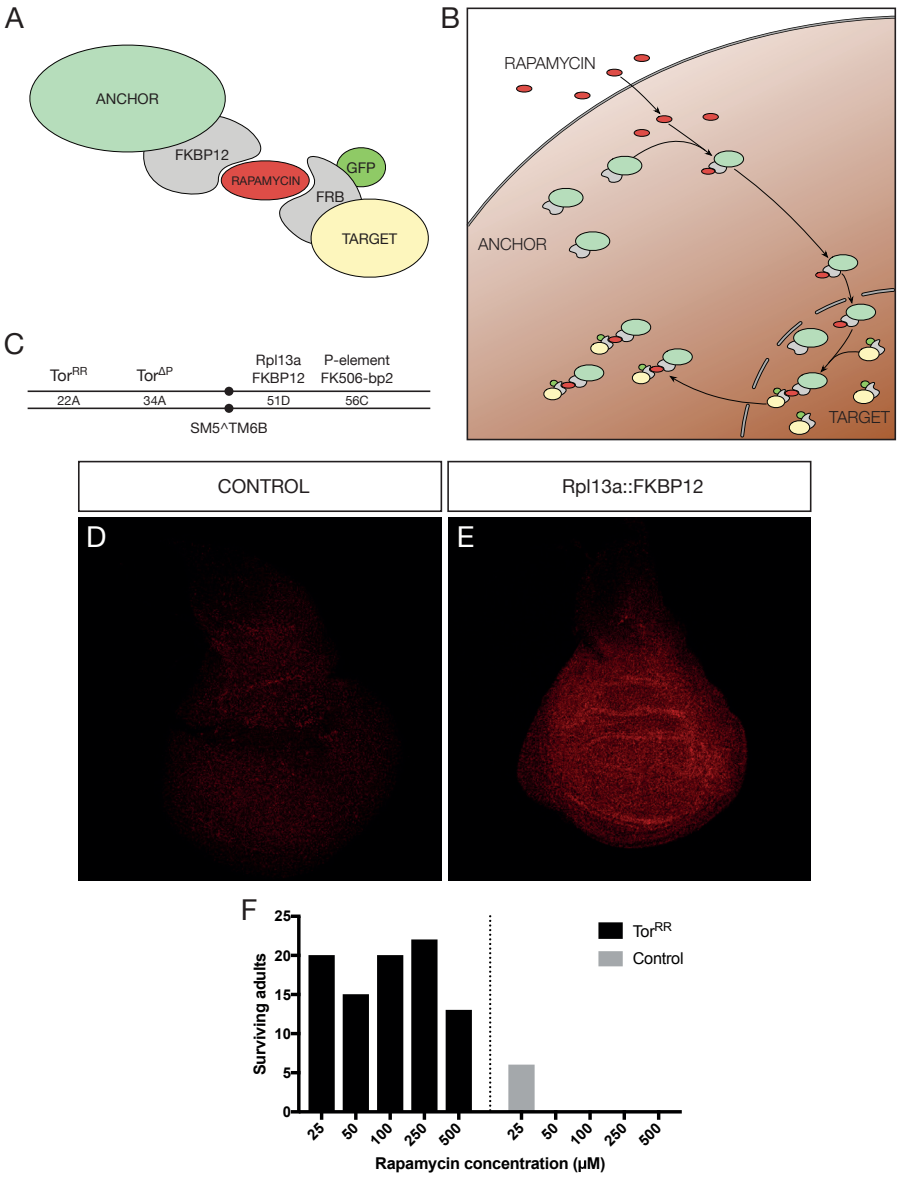

Figure 1. Adapting the anchor-away to *Drosophila*

A) Schematic of the anchor-away components. B) Schematic of the mechanism of action of the anchor-away upon rapamycin addition. The anchor first binds rapamycin, and this complex drives the capture of the target protein to the cytoplasm. C) Chromosomal localization of the anchor-away components in the second chromosome. The components are either homozygous or balanced over SM5^TM6b. D-E) Staining against human FKBP12 in control discs without Rpl13a::FKBP12 (D), or discs from larvae carrying Rpl13a::FKBP12 (E). F) Survival of animals upon different rapamycin treatments with and without Tor^{RR}.

used in the yeast system (Haruki et al. 2008). The protein has an exposed C-terminus and so as in the yeast AA system Rpl13a was tagged with 2 copies of the human FKBP12 rapamycin-binding domain at the C-terminus. These modifications were made in the context of a genomic rescue construct such that the gene was controlled by it endogenous regulatory elements. The construct was integrated in the second chromosome via attB/attP integration (Bischof et al. 2007), and proper localization of the protein was assessed by immunostaining (Fig. 1D-E). Flies carrying all the transgenes were viable. In addition, we generated a transgenic UAS-FKBP12::Rpl13a that could be used to restrict the anchoring to a certain region by using compartment- or tissue-specific Gal4 lines (Fig. 1F).

Once all transgenes were integrated, we assessed if flies were viable

when exposed to rapamycin. We collected eggs in food containing rapamycin, from 20 to 500 μM to determine a concentration that will affect WT larvae but not the rapamycin-resistant AA flies. Based on the viability, we concluded that a concentration of 50 μM rapamycin was optimal (Fig 1F), similar to what has been used in prior studies of Tor signaling in Drosophila (Oldham et al. 2000). Lower amounts of rapamycin allow some WT larvae to develop and very high concentrations affected the transgenic flies, even though they should be resistant to rapamycin.

Generation of anchorable Brinker and Pygopus variants

To test the feasibility of anchoring of nuclear proteins in *Drosophila*, we generated FRB-tagged variant of two well-studied nuclear factors — Brinker (Brk) and Pygopus (Pygo). Brk is the main effector of the Dpp pathway in *Drosophila*. Its expression domain in the wing imaginal disc is restricted to the lateral regions, and a brk LOF produces a clear overgrowth phenotype and derepression of Dpp target genes (Jaźwińska et al., 1999). Pygo is one the binding partner of Armadillo, the fly homolog of β-Catenin. Its recruitment is critical in the signal transduction of Wnt target genes (Kramps et al., 2002). Mutants for pygo present severe undergrowth phenotypes. In addition to the rapamycin binding FRB tag, we also included the eGFP sequence (FRB-GFP), to be able to directly localize the anchored targets. We engineered the targets by using two different approaches.

To generate FRB-GFP::Brk, we introduced the FRB-GFP tag in the N-terminal end of Brk, and cloned it in a BAC construct to integrate the whole genomic fragment via attB/attP in the third chromosome, in the attP-86Fb locus (Bischof et al. 2007), depicted in Fig. 2A. Both expression pattern and subcellular localization of the transgenic protein, assessed by GFP expression, were the same as endogenous Brk (Fig 2D-D'). To assess the efficiency of the anchoring, the transgenic construct was crossed into a line containing the brk^{M68} null allele (Jaźwińska et al. 1999) as well as the other anchor-away transgenes. Anchoring of FRB-GFP::Brk by rapamycin exposure was lethal at the third larval stage. We assessed the effect of Brk sequestering by observing at the phenotypes of third instar larva wing discs. Discs where brk was sequestered at the cytoplasm exhibited overgrow, resembling brk LOF. The effect in Dpp signaling was confirmed by staining against the downstream target Spalt major (Salm). Discs where Brk was anchored showed widespread derepression of Salm, as it is expec-

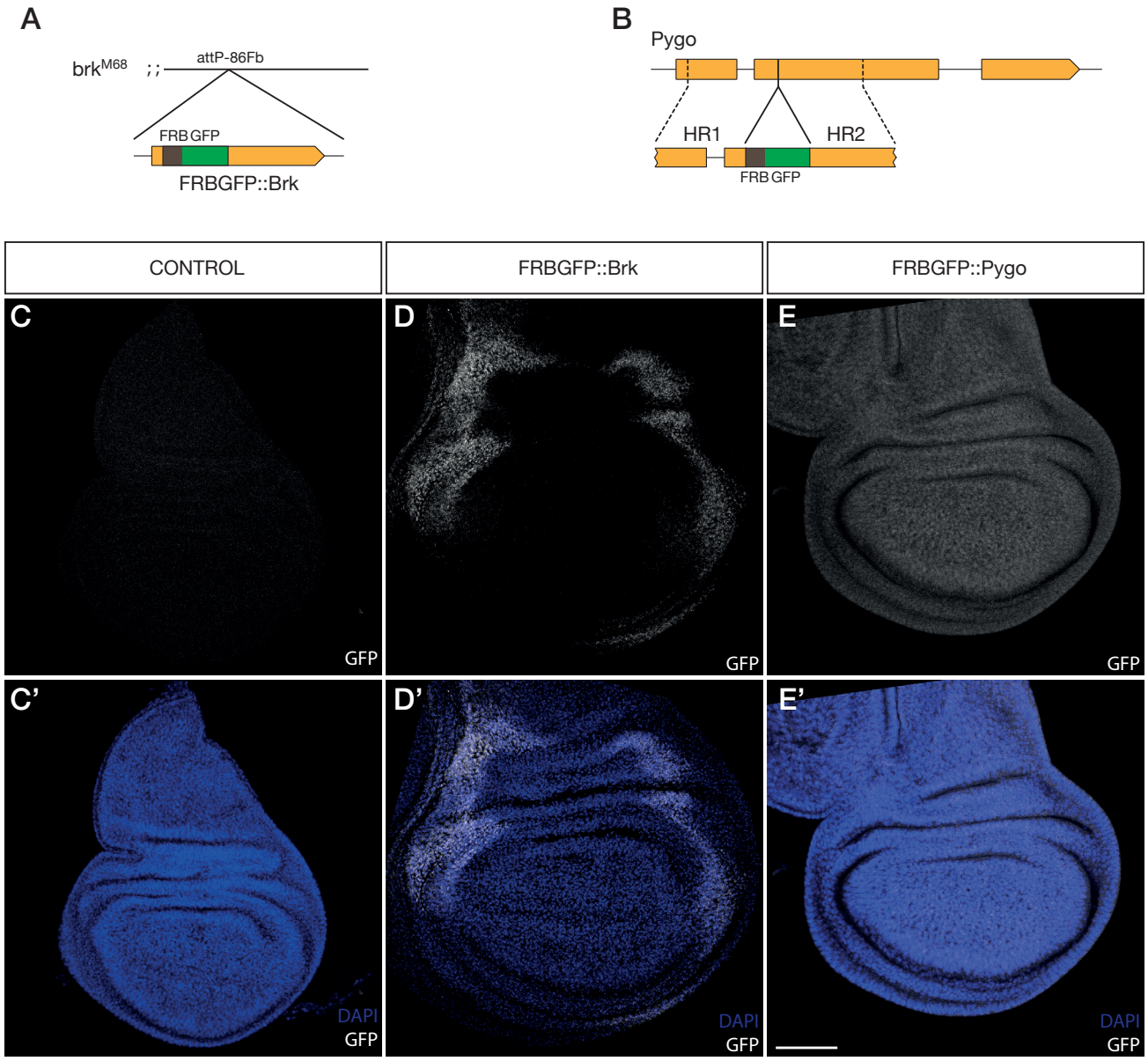

Figure 2. Establishing anchorable Brk and Pygo

A) Schematic of the Brk BAC rescue. This BAC was integrated on the third chromosome and coupled with the null allele brk^{M68}. B) Schematic of the modification of the pygo locus by CRISPR/Cas9. FRBGFP was integrated in frame right after the 5'UTR in the second exon of *pygo*. C-E') Detection of the GFP-tagged targets. C-C') Control discs without anchor-away target. D-D') Discs carrying FRBGFP::Brk over a *brk*-null background. The shape of the disc is normal and Brk is localized in the normal expressing region and is present in the nucleus. E-E') Discs carrying homozygous FRBGFP::Pygo. Discs retain a WT-like shape and Pygo is produced ubiquitously and localizes in the nucleus as expected. Scalebar = 50 µM.

ted in a brk LOF (Suppl. Fig. 1A-B).

To generate a FRB-GFP-tagged Pygo, we used CRISPR/Cas (Port et al. 2014). We recombined the FRB-GFP fragment in frame at the N-terminal region of the endogenous pygo gene (Fig 2B). As with Brk, Pygo expression was unaffected by the modification and showed the expected Pygo localization (Fig 2E-E'). Homozygous FRB-GFP::Pygo flies carrying the an-

chor-away transgenes were exposed to rapamycin. As with FRB-GFP::Brk, anchoring Pygo to the ribosomes resulted in developmental arrest, and larvae were not able to pupariate. To determine the functional consequence of anchoring-away Pygo, we assayed the expression of the Wnt target gene Sensless (Sens) by immunostaining. In larvae fed with rapamycin for 3 days, Sens staining was completely gone, confirming the inactivation of Wnt-target genes (Suppl. Fig. 1C-D).

These results demonstrate that the Anchor-away method works in Drosophila to induce an acute LOF situation.

Anchoring away proteins as a fast and efficient knock-down system

We next wanted to assess how rapidly the anchor-away methods creates a LOF situation.

We first measured how much time does the anchor-away technique needs to generate the LOF effect in dissected wing discs. We used the FRB-GFP::Brk anchoring system and after dissection applied rapamycin-containing Wing Medium 1 (Restrepo et al. 2016). Via colocalization analysis, we found that most Brk was sequestered to the cytoplasm 1 hr after rapamycin treatment, although some protein still colocalized to the nucleus. Extended culturing was problematic and we were therefore unable to assess how long it takes to completely remove Brk from the nucleus *ex vivo*. The culture conditions will be revised to solve this issue.

We also assessed how quickly after feeding larvae with rapamycin did we see relocalization of FRBGFP::Pygo. We collected eggs in regular food, and we let larvae develop until third instar. We then transferred them to rapamycin-containing food, and we dissected them at defined time points. To determine when the effect of Pygo anchoring away affected target genes, we again used Sens levels as a readout. We found that 12 h after treatment Sens protein was not detectable anymore. Pygo detection was also affected. From 6 hrs after rapamycin feeding, there is a decay in FRBGFP::Pygo, with less protein localizing to the nucleus and lower signal. At 12 hrs after treatment, Pygo signal was much lower than the control without treatment. We hypotezise that the decay in signal is due to the sequestering of Pygo to the cytoplasm. As the protein is now more diffuse, the fluorescent signal is also more delocalized and decreases. We cannot exclude, however, that

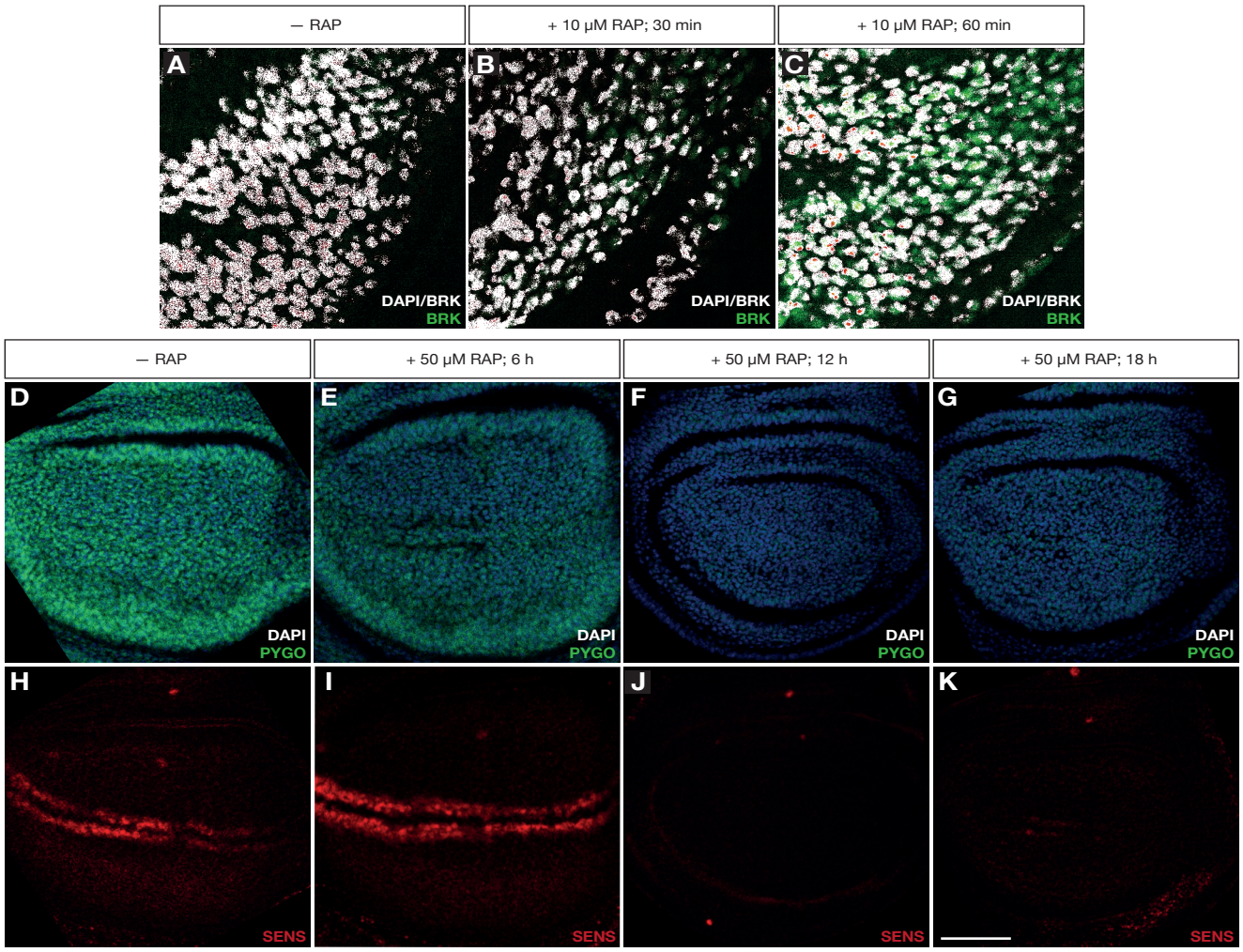

Figure 3. Assesing the timing of the anchoring of Brk and Pygo

A-C) Time lapse of Brk anchoring after rapamycin addition *ex vivo*. Discs were exposed to rapamycin after dissection, and fixed either before rapamycin exposure (A), 30 min after treatment (B) or 60 min after treatment (C). Pictures show colocalization of DAPI and Brk in white and Brk anchored to the cytoplasm in green. D-K) Time lapse of Pygo anchoring after rapamycin treatment *in vivo*. D-G) Pygo and DAPI localization without rapamycin treatment (D), 6 hrs after rapamycin feeding (E), 12 hrs after treatment (F) and 18 hrs after treatment (G). H-K) Sens staining of the same discs as from D-G, to assess inhibition of Pygo activity. Expression of *sens* was completely abolished 12 hrs after treatment. Scalebar = 50 µM.

cytoplasmic anchored Pygo is more easily degraded by the proteasome, causing the decrease of signal. In either case, the effect is detectable as early as 6 hrs after rapamycin feeding, and the pathway is inhibited 12 hrs after treatment.

We show that the Pygo anchoring in vivo works slower as the Brk anchoring *ex vivo*. It remains to determine whether the timing delay in vivo is inherent to the technique, or if the timing depends on the anchored target. The *in vivo* assay presents the advantage of being less invasive and would allow for longer exposures to rapamycin, as an *ex vivo* approach would only allow for short incubation periods of up to 12 hrs (Restrepo et al., 2016).

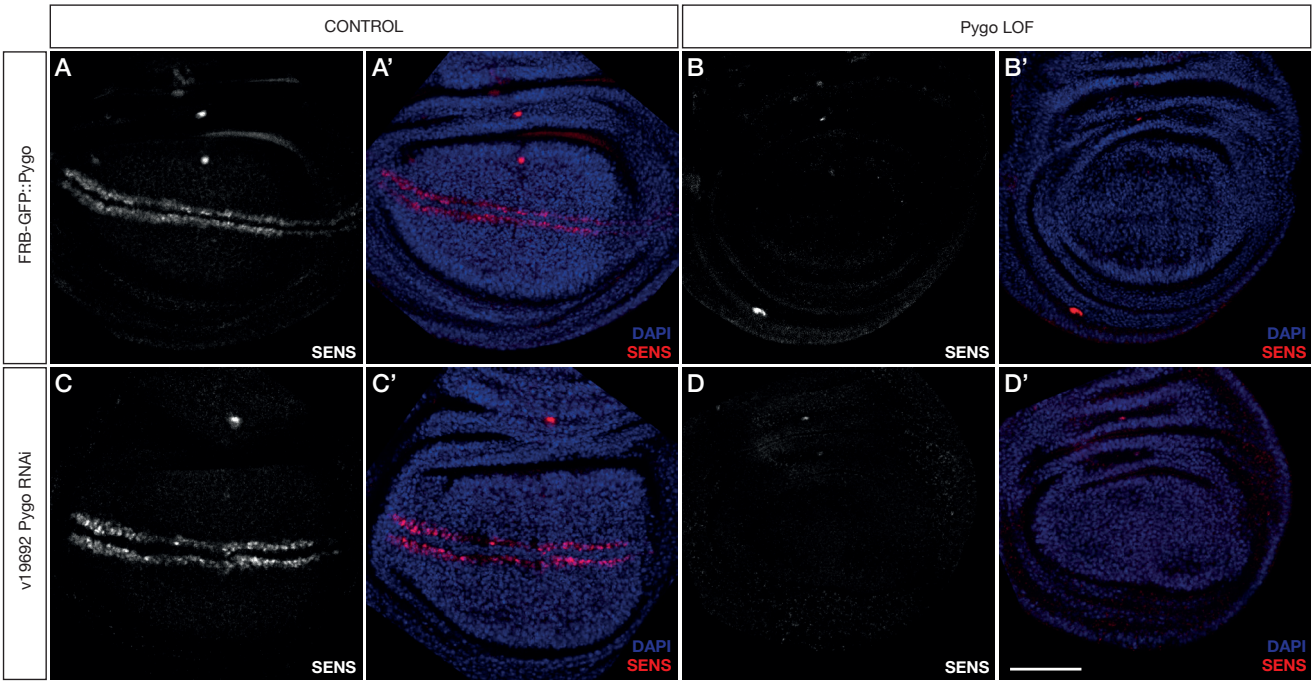

Figure 4. Comparing the anchor-away to RNAi.

A-A') FRBGFP::Pygo discs without rapamycin treatment. B-B') FRBGFP::Pygo discs dissected 48 hrs after rapamycin treatment. C-C') Discs without induction of pygo RNAi. D-D') Discs expressing pygo RNAi during 48 hrs. Scalebar = 50 µM

Depletion via Anchor-away is more efficient and reliable than RNAi downregulation

RNAi-mediated downregulation (Fire et al. 1998) is widely used in *Drosophila*, in part due to the existence of collections targeting all the genes in the genome. Other aspects that have promoted its use are the possibility of spatio-temporal control of the knock-down (Dietzl et al. 2007; Ni et al. 2009). Despite these benefits, RNAi downregulation has drawbacks, such as off-targets that confound analyses and high variability or delayed repression of target (Boutros & Ahringer 2008).

The anchor-away technique is a viable alternative to RNAi, especially for studies of a small number of genes.

One of the major impediments of RNAi is that its efficiency can vary greatly (Boutros & Ahringer 2008). We tested three different RNAi lines which target *pygo* and compared the efficiency of their knock-down functionally examining the effect on by Sens levels (Suppl. Fig. 2). We triggered expression of the RNAi transgene for 48 h. Although all three RNAi were able to decrease pygo levels up to the point where Sens was barely detectable, in some discs Sens levels were still strong, or not affected at all (Suppl.

Fig. 2D,F). This demonstrates the variability inherent in RNAi.

The effects of the Anchor-away system were much more reliable, as Sens staining was never detected after rapamycin treatment in several independent tests of the system (Fig 4B-B'). When RNAi worked, it was as effective as the Anchor-away (Fig. 4D-D', Suppl. Fig. 2B).

With all our data together we present the anchor-away as a very efficient and quick alternative method in *Drosophila* to perform LOF analyses.

Discussion

The Anchor-away technique has been developed in and is widely used in yeast (Haruki et al. 2008; Ding et al. 2014; Tsuchiya et al. 2013). In the current work, we successfully adapted the system to the model organism *Drosophila melanogaster*. The required transgenes were properly expressed, localized in their respective subcellular compartments, and preserved their molecular functions. We confirmed the efficiency of the technique used to trap nuclear proteins. The strength of the LOF phenotypes suggested complete inhibition. The method was also very rapid producing a detectable effect within hours (1 hr ex vivo, 6 hrs in vivo).

RNAi have been useful and will continue to be widely used for its simplicity and amount of lines and libraries available. As a screening technique, it is unmatched thanks to the existing libraries (Dietzl et al. 2007; Ni et al. 2009) and simplicity of use, allowing for quick data acquisition and selection of gene candidates for studies. However, when analyzing specific gene functions, the variability in RNAi has been a constant worry for researchers. Off-targets, or dominant phenotypic effects might alter the results of screens and LOF studies. A big effort has been done over the years to minimize the effects on studies, but they still are a concern (Green et al. 2014; DasGupta et al. 2007). The anchor-away method therefore represents a useful tool to rapidly induce a LOF in *Drosophila*.

Since its discovery, CRISPR/Cas has changed the time we require to generate new alleles. It allows one to quickly generate new transgenic strains, or perform screens in a fraction of the time we could do it before (Hsu et al. 2014). Combined with CRISP/Cas, the anchor-away system can be adapted to any protein target in a time scale of weeks. CRISPR/

Cas has also been used in *Drosophila* to perform genome-wide mutagenesis or overexpression screens (Ewen-Campen et al. 2017; Bassett et al. 2015). Following a similar approach, we could devise a genome-wide application of the anchor-away, where one could perform a genomic CRISPR targeting to generate a library of FRB-tagged proteins, which could be used afterwards for a large-scale study of nuclear factors in Drosophila.

By utilizing different anchors, other proteins could be sequestered from the subcellular compartment where they function. For example, cytoplasmic proteins could be sequestered to the plasma membrane (Tsuchiya et al. 2013). The Anchor-away could also be used to relocate proteins to different compartments and thereby force them to perform a secondary function, providing versatility to the technique. In summary our adaption of the anchor-away system is a useful addition to the toolbox of Drosophilists.

Methods

Drosophila strains

The following fly stocks were used for the experiments: $Tor^{\Delta P}$ and 22A-Tor^{S1956T} (Zhang et al. 2000), FK506-bp2 Kyoto stock 205244 (P{GSV6}GS10737) (Toba et al. 1999), brk^{M68} (Jaźwińska et al. 1999), 56C-Rpl13a::FKBP12, 86Fb-FRBGFP::Brk, FRBGFP::Pygo, Vienna RNAi stocks v19602, v19693, v100724.

Cloning procedures

Rpl13a was tagged with 2xFKBP12 under the control of its own promoter, endogenous 5' and 3' UTRs and supposedly all its endogenous regulatory regions. The resulting transgene was integrated via the ΦC31 integrase system into the landing site at 51D (Bischof et al. 2007).

FRB-GFP::Brk was generated by fusing the FRB-GFP cassette into a BAC containing the whole Brk genomic region by BAC recombineering (Warming et al. 2005). The resulting vector was integrated via the ΦC31 integrase into the landing site at 86Fb (Bischof et al. 2007).

For FRBGFP::Pygo, CRISPR gRNA were cloned in pU6-BbsI-gRNA (Gratz et al. 2013). A donor plasmid (pFRBGFP) was generated by using

the pDsRed-attP plasmid as a backbone. We replaced the fragment between the multiple cloning sites for the homology regions with an in frame FRBGFP DNA fragment, by digesting the plasmid with AarI and SapI and cloning a PCR fragment containing the FRB-GFP fragment in such a way that it will be in frame once the homology arms are cloned in the plasmid. gRNA and donor plasmid were co-injected into embryos expressing nos-Cas9 (F Port et al. 2014). F1 was screened by PCR to confirm the insertion of the FRBGFP fragment in the correct region.

Immunostaining

Third instar imaginal discs were dissected in PBS and fixed during 30 min with 4% Formaldehyde in PBS. Prior to antibody staining, discs were blocked with 2% heat inactivated goat serum (HINGS) and stained overnight with primary antibodies. The following antibodies and concentrations were used: guinea pig α-Sens (Nolo et al. 2000), 1:1000; guinea pig α-Brk (Doumpas et al. 2013), 1:500, Cell Signaling mouse α-FKBP12, 1:500. Secondary antibody staining was performed during 2 hours, using Thermo Fisher Alexa® antibodies. Discs were mounted in Vectashield® and images were taken with a Zeiss™ LSM710 confocal microscope.

Rapamycin culture ex vivo

Imaginal wing discs were dissected in Wing Medium 1 (WM1) (Restrepo et al. 2016), and transferred to reaction tubes. The solution was replaced by WM1 containing rapamycin 50 µM and incubated for 30 min to 2 h. After incubation, Rapamycin was removed and discs were fixed and stained as described in the prior section.

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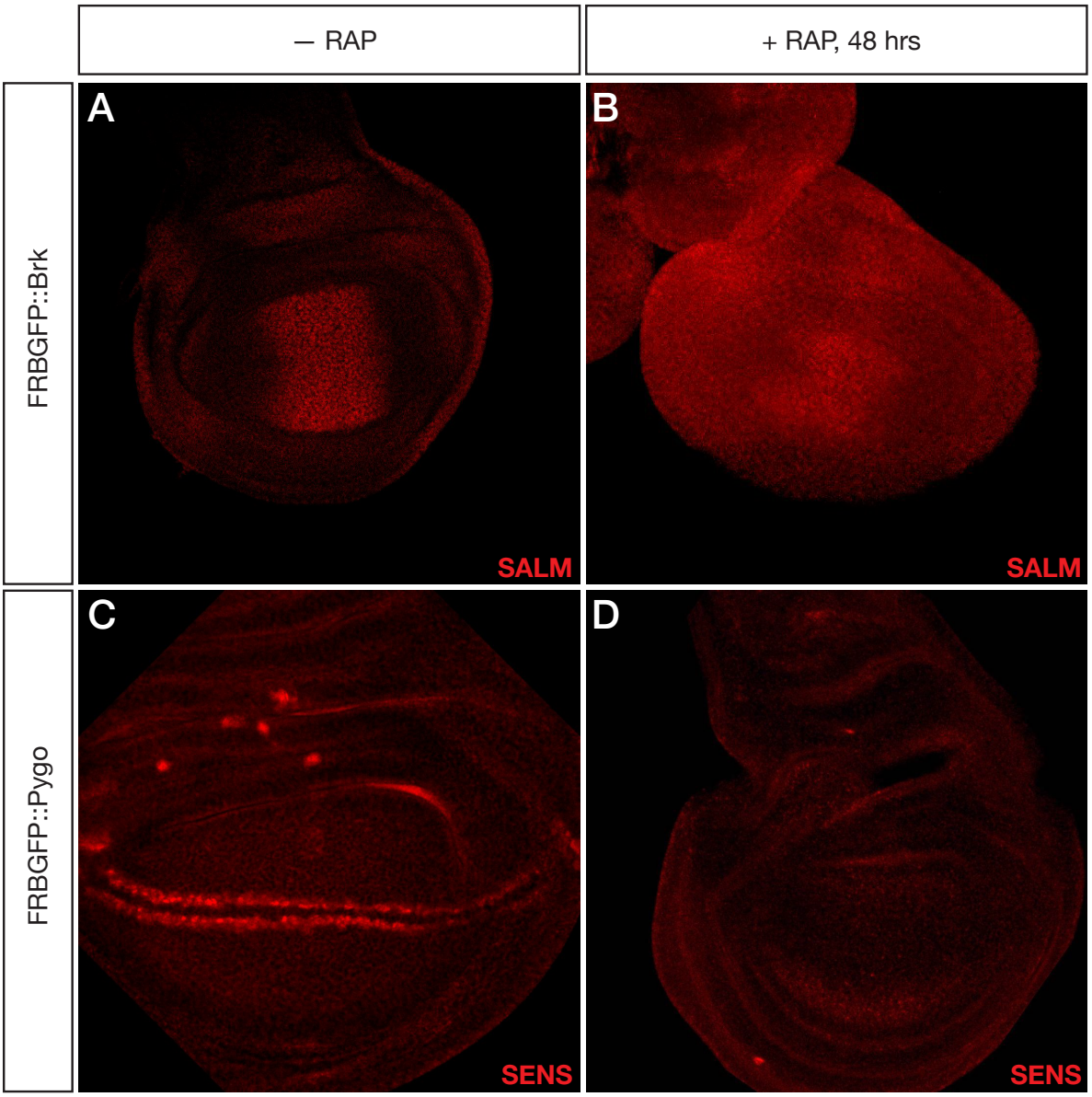
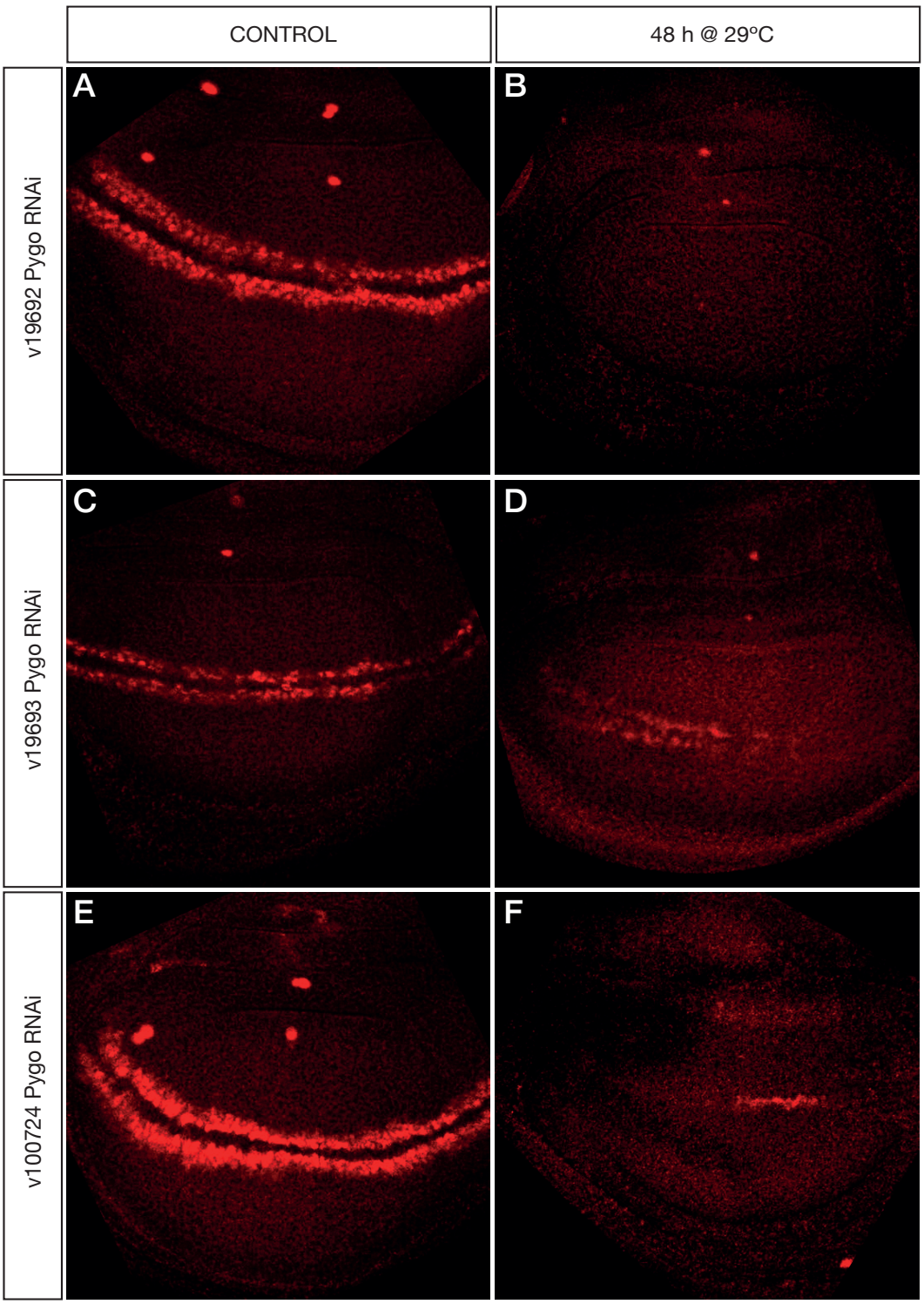
Supplementary material



Supplementary Figure 1. Testing anchoring of Brk and Pygo

A) FRBGFP::Brk disc without rapamycin treatment. B) FRBGFP::Brk disc dissected 48 hrs after feeding rapamycin to larvae. C) Control disc carrying FRBGFP::Pygo, without rapamycin treatment. D) Disc carrying FRBGFP::Pygo dissected 48 hrs after feeding rapamycin to larvae.



Supplementary Figure 2. Testing different Pygo RNAi lines

A-B) RNAi from Vienna line 19692 without (A) and with (B) expression of the RNAi 48 hrs before dissection. C-D) RNAi from Vienna line 19693 wihtout (C) and with (D) RNAi expression 48 hrs before dissection. Notice the localization of residual Sens staining in the anterior region. E-F) RNAi from Vienna line 100724 without (E) and with (F) RNAi expression 48 hrs before dissection. There is residual Sens in the posterior region of the disc.

Chapter 3: Dpp controls growth and patterning in *Drosophila* wing precursors through distinct modes of action

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Dpp controls growth and patterning in Drosophila wing precursors through distinct modes of action

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Abstract Dpp, a member of the BMP family, is a morphogen that specifies positional information in *Drosophila* wing precursors. In this tissue, Dpp expressed along the anterior-posterior boundary forms a concentration gradient that controls the expression domains of target genes, which in turn specify the position of wing veins. Dpp also promotes growth in this tissue. The relationship between the spatio-temporal profile of Dpp signalling and growth has been the subject of debate, which has intensified recently with the suggestion that the stripe of Dpp is dispensable for growth. With two independent conditional alleles of *dpp*, we find that the stripe of Dpp is essential for wing growth. We then show that this requirement, but not patterning, can be fulfilled by uniform, low level, Dpp expression. Thus, the stripe of Dpp ensures that signalling remains above a pro-growth threshold, while at the same time generating a gradient that patterns cell fates.

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Introduction

During development, tissue growth must be precisely coupled with patterning to ensure that the right number of cells can contribute to the various substructures within each organ (***Restrepo et al., 2014***) (***Baena-Lopez et al., 2012***; ***Bryant and Gardiner, 2016***; ***Hariharan, 2015***; ***Irvine and Harvey, 2015***; ***Johnston and Gallant, 2002***; ***Wartlick et al., 2011a***). Not surprisingly, many signalling molecules that specify positional information also control growth (***Baena-Lopez et al., 2012***; ***Restrepo et al., 2014***). This has been particularly well demonstrated in *Drosophila* wing imaginal discs, epithelial pockets that grow during larval stages and eventually give rise to the wing proper, the wing hinge and a part of the thorax called the notum (***Figure 1A***). Segregation of wing imaginal discs into the territories that give rise to these three structures is controlled by a series of signalling events involving EGFR, JAK/STAT, Notch, and Hedgehog signalling, culminating in sustained expression of Wingless and Dpp in orthogonal stripes until the end of the third instar (***Blackman et al., 1991***; ***Neumann and Cohen, 1996***; ***Zecca et al., 1995***). Both Wingless and Dpp are essential for growth (***Baena-Lopez et al., 2009***; ***Burke and Basler, 1996***; ***Restrepo et al., 2014***; ***Spencer et al., 1982***; ***Wartlick et al., 2011b***). Here, we focus on the role of Dpp, which is expressed along the anterior-posterior (A/P) compartment boundary in a pattern that cuts across the prospective notum, hinge and wing proper (***Figure 1A***). We look specifically at the prospective wing, which forms from a central region of the disc called the pouch. A wide range of evidence suggests that, in this region, Dpp acts as a morphogen. Graded distribution of the endogenous protein has not been directly visualized for lack of a suitable antibody against the mature secreted protein. However, the nested pattern of expression of target genes and the patterning activity of ectopic Dpp are strongly indicative of graded signalling activity (***Lecuit et al., 1996***; ***Nellen et al., 1996***; ***Schwank and Basler,***

eLife digest From the wings of a butterfly to the fingers of a human hand, living tissues often have complex and intricate patterns. Developmental biologists have long been fascinated by the signals – called morphogens – that guide how these kinds of pattern develop. Morphogens are substances that are produced by groups of cells and spread to the rest of the tissue to form a gradient. Depending on where they sit along this gradient, cells in the tissue activate different sets of genes, and the resulting pattern of gene activity ultimately defines the position of the different parts of the tissue.

Decades worth of studies into how limbs develop in animals from mice to fruit flies have revealed common principles of morphogen gradients that regulate the development of tissue patterns. Morphogens have been shown to help regulate the growth of tissues in a number of different animals as well. However, how the morphogens regulate tissue size and what role their gradients play in this process remain topics of intense debate in the field of developmental biology.

In the developing wing of a fruit fly, a morphogen called Dpp is expressed in a thin stripe located in the centre and spreads to the rest of the tissue to form a gradient. Bosch, Ziukaite, Alexandre et al. have now characterised where and when the Dpp morphogen must be produced to regulate both the final size of the fly's wing and the number of cells the wing eventually contains. The experiments involved preventing the production of Dpp in the developing wing in specific cells and at specific stages of development. This approach confirmed that Dpp must be produced in the central stripe for the wing to grow. Matsuda and Affolter and, independently, Barrio and Milaín report the same findings in two related studies. Moreover, Bosch et al. and Barrio and Milaín also conclude that the gradient of Dpp throughout the wing is not required for growth.

Further work will be needed to explain how the Dpp signal regulates the growth of the wing. The answer to this question will contribute to a better understanding of the role of morphogens in regulating the size of human organs and how a failure to do so might cause developmental disorders.

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2010; ***Zecca et al., 1995***) which is high around the A/P boundary, low further away, and undetectable at the lateral edges of the disc. High signalling activity, within and around the stripe of Dpp expression, is marked by immunoreactivity against phosphorylated Mad (P-Mad) and the expression of *spalt-major* (*salm*) while low signalling activity suffices to activate *optomotor blind* (*omb*) expression over a wider area of the prospective wing (***Burke and Basler, 1996***; ***Lecuit et al., 1996***; ***Nellen et al., 1996***; ***Tanimoto et al., 2000***). In wing imaginal discs, Dpp signalling controls gene expression indirectly, through repression of a transcriptional repressor encoded by the *brinker* gene (***Martín et al., 2004***). Thus, the inverse gradient of Brinker expression provides yet another means of detecting Dpp signaling activity (***Schwank et al., 2008***).

As a morphogen, Dpp is a pattern organiser. For example, graded Dpp signalling determines the position of wing veins, particularly veins 2 and 5, through regulation of *salm* and *omb* (***Campbell and Tomlinson, 1999***; ***Jaźwińska et al., 1999***; ***Minami et al., 1999***). Dpp also clearly contributes to growth. Indeed, in the absence of Dpp signalling, wings (and other appendages) fail to grow (***Bangi and Wharton, 2006***; ***Restrepo et al., 2014***; ***Spencer et al., 1982***). The pro-growth role of Dpp is in part mediated through regulation of Myc (***Doumpas et al., 2013***), although a comprehensive understanding of growth regulation by Dpp signalling remains lacking. In wild-type imaginal discs, proliferation is approximately uniform while Dpp signalling is graded. Therefore, there is no apparent correlation between the level of Dpp signalling and the growth rate. How does a graded signal trigger a uniform response? Experiments involving the creation of abrupt differences in signalling suggested that local differences in Dpp signalling activity, that is, the spatial gradient of signalling, could be the trigger of growth (***Rogulja and Irvine, 2005***). This would provide an elegant mechanism for growth termination as the gradient would be expected to become shallower during growth (***Day and Lawrence, 2000***). However, there is no evidence that smooth differences in signalling activity associated with the endogenous gradient control growth. An alternative model is that the temporal gradient (the local relative increase in signalling activity) could be the trigger of

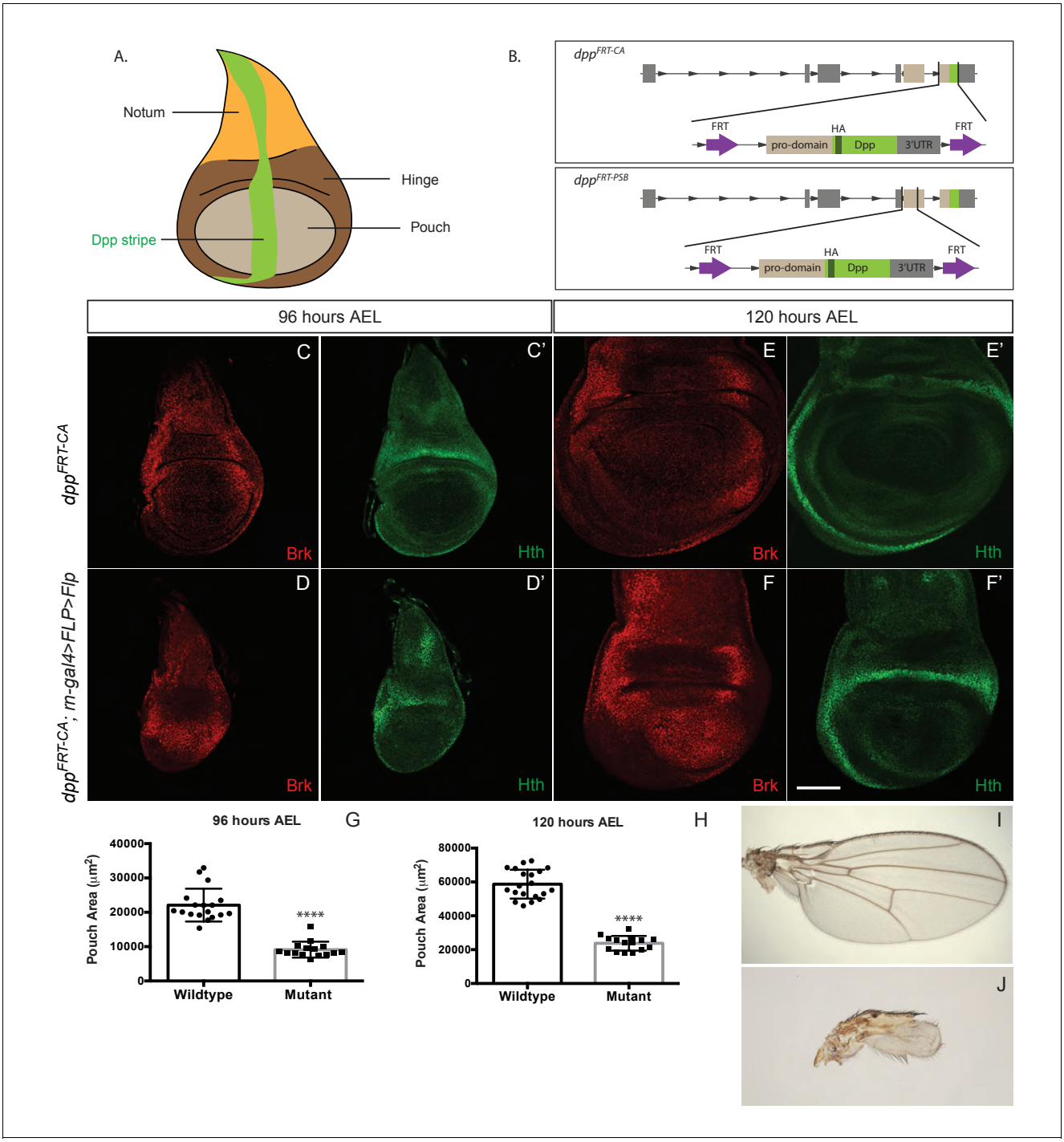

Figure 1. Growth of the prospective wing requires Dpp expression within the pouch. (**A**) Diagram highlighting the three domains of wing imaginal discs and the stripe of Dpp expression. (**B**) Diagram of the two conditional alleles we created, showing the region deleted from the genome and the inserted fragment. (**C–F'**). Inactivation of *dpp*$^{FRT\text{-}CA}$ in the pouch (with *rotund-gal4 UAS-Flp*) leads to derepression of *brinker* and reduced growth (shown here in discs fixed at 96 hr and 120 hr AEL). The edge of the pouch is marked by the weak inner ring of Hth expression. However, since the outer ring is more readily visible, this is the marker we used to measure pouch size (thus overestimating). (**G, H**) Quantification of the area enclosed by the Hth outer ring at the two stages (each dot/square represents one imaginal disc). (**I, J**) Wings from control (**I**) and experimental (**J**) adults. The scale bar, which

Figure 1 continued on next page

Figure 1 continued

represents 50 μm, applies to panels (**C-F'**). In panels (**G** and **H**) statistical significance of the difference between experimental and control samples was assessed with Student's t-test, assuming equal variance and a Gaussian distribution ($p<0.0001$).

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The following source data and figure supplement are available for figure 1:

Source data 1. Pouch area.

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Figure supplement 1. Inactivation of Dpp specifically in the pouch.

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proliferation (***Wartlick et al., 2011b***), a model that has also been questioned (***Harmansa et al., 2015***; ***Schwank et al., 2012***).

In agreement with the notion that Dpp controls growth through repression of *brinker*, imaginal discs lacking both Dpp and Brinker proliferate extensively (***Martín et al., 2004***; ***Schwank et al., 2008***). Importantly, only the lateral region of the pouch (as well as the prospective hinge) overproliferates, while the medial area proliferate normally. Thus, depending on the distance from the stripe of Dpp, the cells of the pouch have a different propensity to proliferate. The main role of the Dpp/Brinker system would be to equalize this difference (***Schwank et al., 2008***). Thus, the inherent tendency of lateral cells to proliferate is slowed down by Brinker, while in medial cells Dpp emanating from its central stripe prevents Brinker-mediated suppression of growth.

Despite strong evidence in support of the above model, Akiyama and Gibson recently suggested that the central stripe of Dpp expression is dispensable for wing growth, and that the prospective pouch requires a source of Dpp in the anterior compartment to achieve growth (***Akiyama and Gibson, 2015***). To control Dpp activity, these authors created a conditional *dpp* allele (here referred to as $dpp^{FRT\text{-}TA}$) by deleting an essential exon and replacing it with a rescuing fragment flanked by Flp Recombination Targets (FRTs). They found that inactivation of this allele at the A/P compartmental boundary in the center of the medial region, had no adverse effect on growth. Inactivation was deemed effective within the pouch because no immunoreactivity against pro-Dpp was detectable there. This led the authors to conclude that the central stripe of Dpp, from where the Dpp gradient originates, is not required for growth. To account for the continued growth observed in the absence of the Dpp stripe, they suggest that perhaps low level Dpp originating from the anterior compartment could suffice to promote growth in the pouch. Here we show, with two new validated conditional alleles, that deletion of the central stripe of Dpp is deleterious to growth. We then investigate and compare the requirements of Dpp within the pouch for growth versus patterning.

Results

Wing growth requires Dpp expression in the prospective wing

To generate means of reliably controlling Dpp activity, we devised two conditional *dpp* alleles, $dpp^{FRT\text{-}CA}$ and $dpp^{FRT\text{-}PSB}$, that can be inactivated by Flp (***Figure 1B***). In both cases, hemaglutinin (HA) tags were included to enable detection of endogenously produced mature Dpp. Flp was then expressed in various patterns to trigger excision of the essential exon. First, Dpp production was inactivated throughout the prospective wing either with *rotund-gal4* and *UAS-Flp* in homozygous $dpp^{FRT\text{-}CA}$ or with *nubbin*-Gal4 and *UAS-Flp* in homozygous $dpp^{FRT\text{-}PSB}$. No HA immunoreactivity (HA-Dpp) could be detected in the pouch from 96 hr after egg laying (AEL) onward (***Figure 1—figure supplement 1***), indicating efficient gene inactivation. HA (i.e. Dpp) was still detectable in the prospective hinge and notum, as expected since Gal4 activity was mostly confined to the pouch. Immunostaining with anti-Brinker showed that *brinker* expression was derepressed throughout the pouch (***Figure 1C–F*** and ***Figure 1—figure supplement 1***), confirming that Dpp signalling was eliminated there. Note that the down-regulation of Brinker around residual Dpp expression in the hinge did not extend into the pouch (arrowhead in ***Figure 1—figure supplement 1D***), suggesting that Dpp produced in the hinge has little effect on gene expression in the pouch. In both experiments, growth was markedly impaired, an effect that was quantified for $dpp^{FRT\text{-}CA}$ by marking the edge of the pouch with anti-Homothorax (anti-Hth) (***Azpiazu and Morata, 2000***; ***Casares and Mann, 2000***)

and measuring the enclosed area at 96 and 120 hr AEL (***Figure 1G–H***). The pouch of experimental discs (*dpp^{FRT-CA}; rotund-Gal4, UAS-Flp*) was significantly smaller than that of their wild-type siblings at equivalent stages. It was, however, not completely eradicated, perhaps because of delayed *dpp* inactivation or residual BMP signalling by *glass bottom boat* (*gbb*) (***Ray and Wharton, 2001***). Since the *dpp^{FRT-CA}; rotund-Gal4, UAS-Flp* genotype is viable, the growth deficiency was also readily apparent in the adults that emerged (***Figure 1I,J***). These results confirm that production of mature Dpp within the pouch is required for this tissue to grow and that Dpp originating from outside the pouch does not compensate.

Temporal requirement of dpp for wing growth

To assess whether Dpp is continuously required for wing growth, we first inactivated *dpp$^{FRT-PSB}$* at different times by Flp expressed from a *hsp70-Flp* transgene. Larvae were heat shocked at 48, 72 and 96 hr AEL and wing imaginal discs were fixed at 120 hr AEL. Staining with anti-HA confirmed the efficiency of gene inactivation although occasional spots of residual HA-Dpp expressing cells could be detected (***Figure 2***). Inactivation of *dpp* at 48 and 72 hr AEL resulted in widespread derepression of *brinker*, confirming the impairment in Dpp signalling. Heat shocking at 48 and 72 hr AEL resulted in markedly reduced growth, while later excision (96 hr AEL) had a milder effect. The

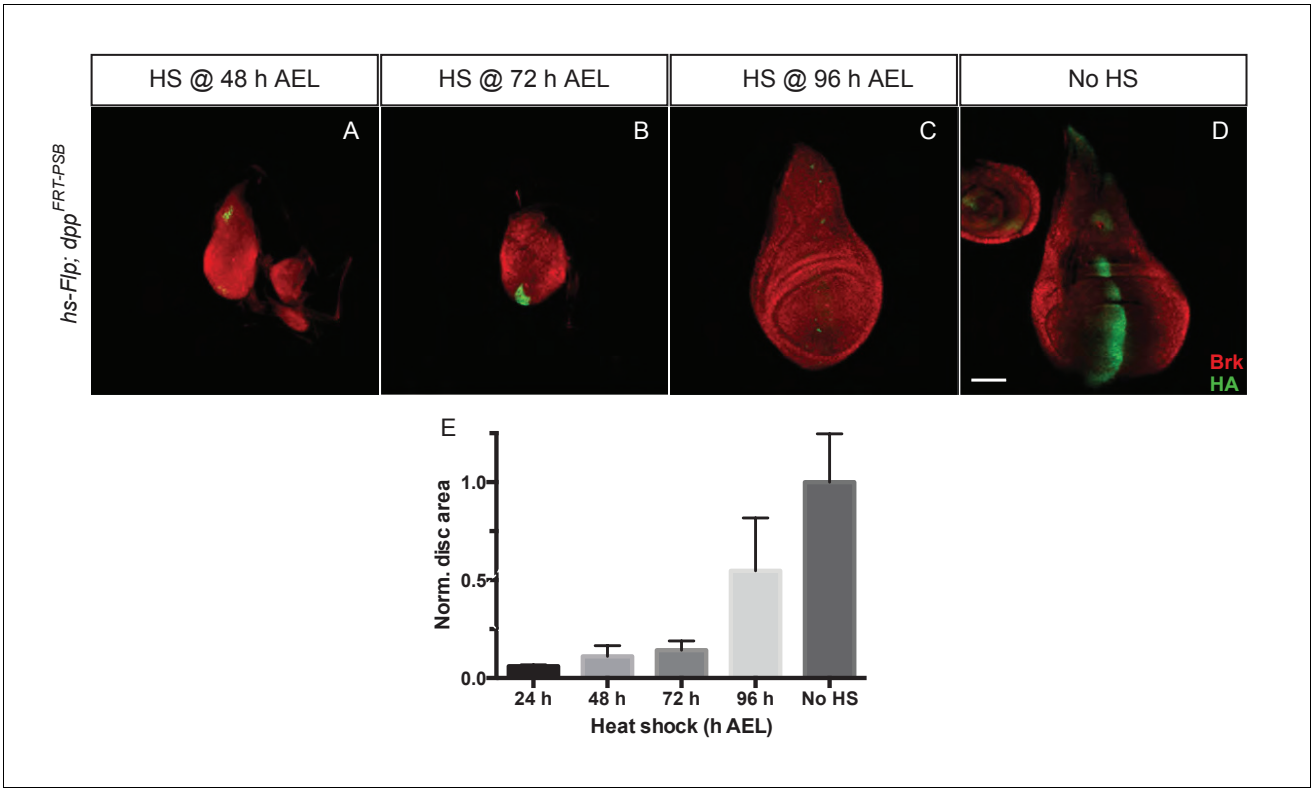

Figure 2. Temporal requirement of Dpp for growth. (**A–D**) Imaginal discs at 120 hr AEL following inactivation of *dpp$^{FRT-PSB}$* by induction of *hsp70-Flp* at the indicated times. Inactivation of Dpp leads to ubiquitous derepression of *brinker*, with the exception of residual HA-Dpp expressing clones (Representative examples are shown). (**E**) The total surface area of discs heat shocked at 24, 48, 72, and 96 hr AEL was measured and normalised to the average surface area of control discs (n = 4 for 24 hr AEL and n = 20 for the other time points). Area measurement for each time point was compared to the control area (no heat shock) with a one-way ANOVA. The p value was highly significant (<0.0001) for every side by side comparison except for 96 hr AEL vs 120 hr AEL. Scale bar = 50 μm.

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The following source data is available for figure 2:

Source data 1. Total disc area.

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relatively weak impact of heat shocks at 96 hr could be due to perdurance of Dpp or downstream events. Alternatively, any effect on growth might be hard to detect beyond this time because the growth rate of imaginal discs decreases with age (**Johnston and Sanders, 2003**). We conclude that the results of timed inactivation experiments show that Dpp must be continuously produced at least up to 96 hr, perhaps beyond, for the prospective wing to grow.

Wing growth requires the endogenous stripe of dpp expression

Our findings so far indicate that Dpp must be produced in the pouch and during the 48–96 hr AEL period in order for the wing to grow. In this region, the major expression domain of Dpp is in a stripe along the A/P boundary (**Masucci et al., 1990**). It is therefore expected that, as shown in **Figure 3**, inactivation of Dpp specifically in this stripe would eradicate Dpp expression in the pouch and lead to growth impairment. Surprisingly, inactivation of $dpp^{FRT\text{-}TA}$ with Flp expressed under the control of *dpp-Gal4* ($dpp^{FRT\text{-}TA}$ dpp^{BLK}*-Gal4 UAS-Flp*) was reported to have no adverse effect on growth (**Akiyama and Gibson, 2015**). In this genetic background, expression of *salm* and *omb* was disrupted, indicating that Dpp production was indeed impaired. It was therefore suggested that the stripe of Dpp expression may not be needed for growth because of the existence of another source of Dpp outside the stripe (**Akiyama and Gibson, 2015**). Indeed, long-term lineage tracing by G-TRACE suggests that progenitors of cells located anterior to the stripe could express Dpp (**Evans et al., 2009**), at least at some point during development. To gain further information on the pattern of *dpp* expression in the wing pouch, we created a reporter line ($dpp^{FRT\text{-}REP}$) expressing the readily detectable marker CD8-GFP from the endogenous *dpp* locus. An excisable cassette expressing Dpp was included upstream of the CD8-GFP coding sequences (**Figure 3—figure supplement 1A**) to allow expression of functional Dpp during embryogenesis, which requires two functional alleles. Thus, during embryogenesis, CD8-GFP is not expressed and the two alleles produce wild-type Dpp. Only after expression of Flp does this allele act as a reporter, in the domain of Flp expression. Cassette excision was induced after embryogenesis with *rotund-Gal4* and *UAS*-Flp, making CD8-GFP a reporter of *dpp* transcription in the pouch. At 72, 96 and 120 hr AEL, GFP was only detectable along the A/P boundary (**Figure 3—figure supplement 1B–D**). Thus, anterior to the stripe, the activity of the *dpp* promoter must either be very low or take place before 72 hr AEL. Therefore, it is unlikely to promote growth, at least after this time period. This conclusion spurred us to re-assess the role of the Dpp stripe in growth.

We tested the role of the endogenous stripe of Dpp in wing growth by inactivating our conditional alleles with *UAS-Flp* and *dpp-Gal4*. To enable comparison with the results of Akiyama and Gibson (**Akiyama and Gibson, 2015**), we chose the same dpp^{BLK}*-Gal4* transgene (**Staehling-Hampton et al., 1995**). This strain was generated many years ago and kept separately in our respective laboratories. We therefore characterised the different dpp^{BLK}*-Gal4* lines by splinkerette PCR (**Potter and Luo, 2010**). Although the three stocks displayed sequence polymorphisms, they all carried the dpp^{BLK}*-Gal4* transgene at the same location, confirming that they all originated from the same initial stock and could be used interchangeably (**Figure 3—figure supplement 2**). The dpp^{BLK}*-Gal4 UAS-Flp* combination was introduced in $dpp^{FRT\text{-}CA}$ and $dpp^{FRT\text{-}PSB}$ homozygotes to inactivate *dpp* within the stripe. In both cases, efficiency of excision was assessed by staining imaginal discs with anti-HA, which marks functional, mature Dpp in the unexcised alleles. At 96 hr AEL, HA immunoreactivity was eliminated from the whole disc, except in a previously characterised zone located outside of the pouch, in the posterior prospective hinge (**Foronda et al., 2009**) (arrowhead in **Figure 3B,D** and **Figure 3—figure supplement 3C,D**). Such residual expression is reproducible and likely represents an area where dpp^{BLK}*-Gal4* does not recapitulate the endogenous Dpp expression domain, as noted previously (**Akiyama and Gibson, 2015**). However, in the rest of the disc, including the whole pouch, the dpp^{BLK}*-Gal4 UAS-Flp* combination appeared to trigger efficient recombination and hence inactivation of *dpp*. Importantly, this was associated with derepression of *brinker* (**Figure 3B,D**) and a marked reduction (84%) of pouch size at the end of the growth period (**Figure 3G** and **Figure 3—figure supplement 3E**).

The lack of growth noted above is in contrast with the report that $dpp^{FRT\text{-}TA}$ dpp^{BLK}*-Gal4 UAS-Flp* imaginal discs attain a normal size and express Brinker throughout the pouch at 120 hr AEL (**Akiyama and Gibson, 2015**). This is in stark contradiction with the model that Dpp stimulates growth through repression of Brinker and that Brinker expression in the pouch is incompatible with growth (**Schwank et al., 2008**). To investigate this apparent inconsistency, we re-examined $dpp^{FRT\text{-}}$

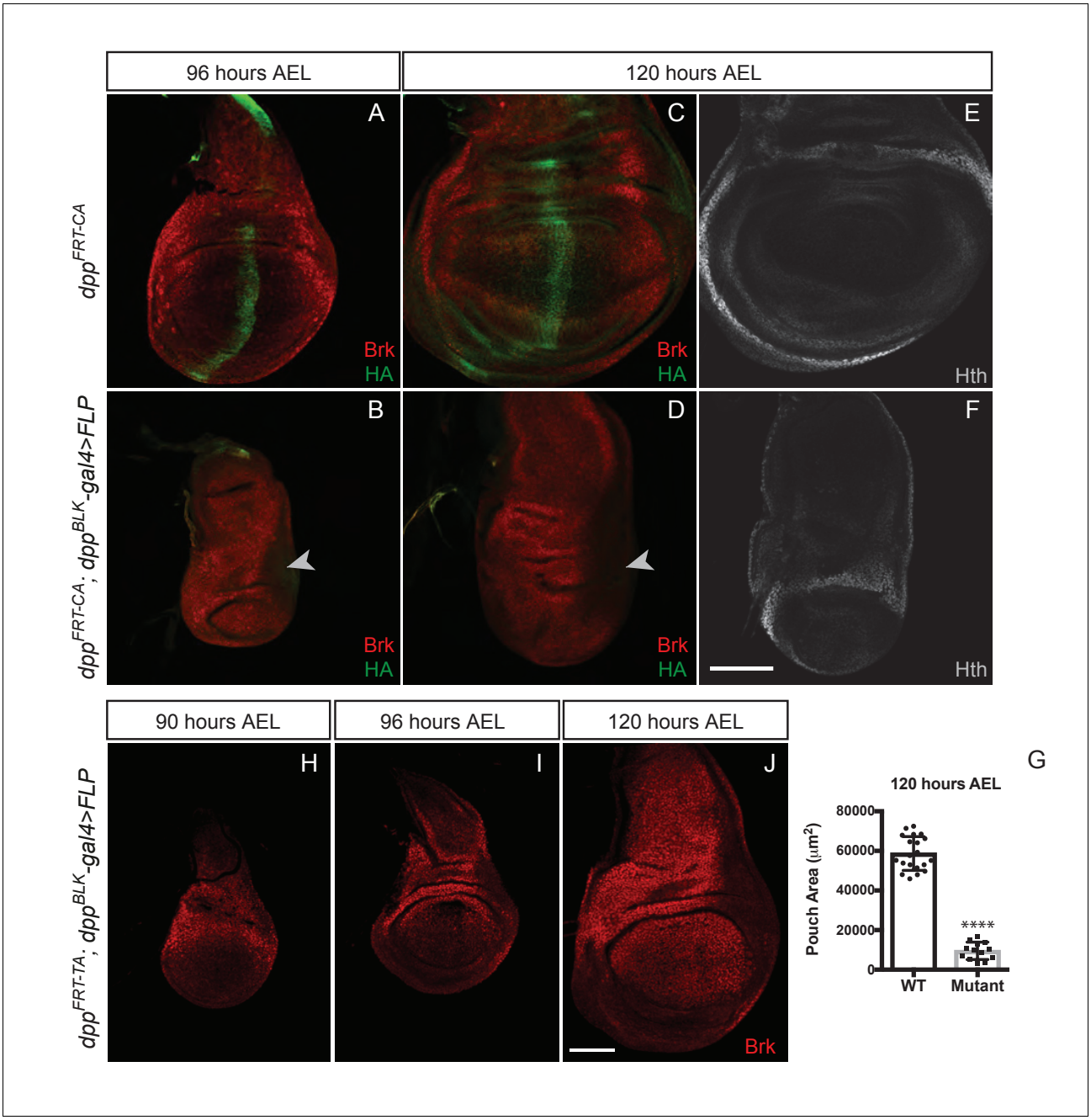

Figure 3. Growth of the prospective wing requires the endogenous stripe of Dpp expression. (**A–F**) Inactivation of *dpp*$^{FRT\text{-}CA}$ in the normal domain of Dpp expression (with *dpp*^{BLK}*-Gal4 UAS-Flp*) leads to depression of *brinker* and reduced growth (shown here in discs fixed at 96 and 120 hr AEL). A zone of *brinker* repression can be seen in the prospective hinge around weak residual Dpp expression (arrowhead in **B**, **D**). (**G**) Quantification of the pouch area (area enclosed by the outer ring of Hth) in control and experimental discs (each dot/square represents a disc). Asterisks in panels G denote the statistical significance of the difference between experimental and control samples, using Student's t-test, assuming equal variance and a Gaussian distribution. (**H–J**) Inactivation of *dpp*$^{FRT\text{-}TA}$ in the normal domain of Dpp expression (with *dpp*^{BLK}*-Gal4 UAS-Flp*) only leads to Brinker derepression after growth has taken place. At earlier stages (90 and 96 hr AEL), Brinker is repressed, indicating residual Dpp signaling activity. Scale bar = 50 μm.
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The following source data and figure supplements are available for figure 3:

Figure 3 continued on next page

Figure 3 continued

Source data 1. Pouch area.
DOI: 10.7554/eLife.22546.009
Figure supplement 1. A reporter inserted at the locus shows that *dpp* expression is confined to the stripe along the A/P boundary.
DOI: 10.7554/eLife.22546.010
Figure supplement 2. Comparison of various *dpp-Gal4* strains.
DOI: 10.7554/eLife.22546.011
Figure supplement 3. Inactivation of $dpp^{FRT\text{-}PSB}$ in the domain of *dpp* expression abolishes growth.
DOI: 10.7554/eLife.22546.012

^{TA} dpp^{BLK}*-Gal4 UAS-Flp* imaginal discs, not only at 120 hr AEL but also at earlier stages. We confirmed that the discs attain a normal size and express Brinker at 120 hr AEL (***Figure 3J***). However, at 90 and 96 hr AEL, during the growth phase, Brinker was repressed within the pouch (***Figure 3H,I***), a clear indication that Dpp signalling is still active at these stages. We suggest that, in this genotype, Dpp signalling is eradicated but only after most growth has taken place. These results suggest that the TA allele may not be as readily inactivated by dpp^{BLK}*-Gal4 UAS-Flp* as the PSB and CA alleles.

The efficacy of gene inactivation was assessed for all three alleles by expressing Flp from a *hs-Flp* transgene under identical heat-shock conditions and measuring *brinker* expression by qRT-PCR. The results show that *brinker* expression was derepressed in all cases but less so with $dpp^{FRT\text{-}TA}$ than with $dpp^{FRT\text{-}PSB}$ and $dpp^{FRT\text{-}CA}$ (***Figure 4A***). These results indicate that $dpp^{FRT\text{-}TA}$ is less readily excised than the other two alleles. Allele 'excisability' was also assessed functionally by measuring imaginal disc size following heat-shock-induced expression of Flp at different times (***Figure 4B–K***). Growth was impaired in a more pronounced manner with $dpp^{FRT\text{-}PSB}$ and $dpp^{FRT\text{-}CA}$ than with $dpp^{FRT\text{-}TA}$, especially with a heat shock at 72 hr AEL, a time when inactivation of Dpp signalling has a strong effect on growth (see quantification in ***Figure 4K***). Therefore, molecular and functional assays suggest that the $dpp^{FRT\text{-}TA}$ allele may not be as readily inactivated as our alleles, perhaps because of differences of sequence context around the FRT sites. We note that one of the FRTs of $dpp^{FRT\text{-}TA}$ is flanked by a LoxP site, which could conceivably impair recombination. In any case, our results show that precluding striped expression of Dpp along the A/P boundary does interfere with wing growth.

Uniform Dpp expression suffices for growth but not patterning

Our results so far show that Dpp expression from the endogenous stripe is required for the growth of wing precursors. They do not address, however, whether a spatial or temporal gradient is necessary. To investigate this question, we took advantage of our conditional alleles to eliminate endogenous *dpp* expression while at the same time inducing uniform constant expression from a transgene. The *rotund-Gal4* and *UAS-Flp* combination was used to simultaneously excise the FRT cassettes of $dpp^{FRT\text{-}CA}$ and *Tubα1-FRT-f*$^{+}$*-FRT-dpp*, a transgene previously shown to trigger intermediate signalling activity, sufficient to activate *omb* but not *salm* expression (***Zecca et al., 1995***). As expected, in the resulting 'rescued' discs, Omb was expressed uniformly, although at a reduced level and Brinker was repressed. (***Figure 5A–D***). However, pMad immunoreactivity was at the low level normally seen in the lateral region (***Figure 5E,F***), suggesting that the level of signalling achieved by *Tubα1-dpp* is similar to that present far from the normal stripe of Dpp. About half the discs of this genotype reached an approximately normal size at the end of the third instar while the other half overgrew slightly (as is the case for the disc shown in ***Figure 5B***). Sustained growth was confirmed by assessing proliferation rates with anti-pH3 staining of discs dissected from late larvae crawling in the food. As shown in ***Figure 5I–L***, 'rescued' and wild-type discs proliferated at approximately the same rate while discs lacking *dpp* proliferated at a lower rate in the pouch area. This result suggests that uniform and constant Dpp signalling is sufficient to promote growth in the pouch. It also suggests that the level of signalling needed to promote growth is much lower than that needed to produce peak p-Mad immunoreactivity.

Since veins form at stereotypical positions in Drosophila wings, they provide a convenient marker of patterning. The five longitudinal veins are distinctly specified by various signalling pathways (reviewed in [***Blair, 2007***]). Most relevant for this paper, the positioning of veins 2 and 5 is dependent on Dpp signalling. Prospective veins can be recognised in late imaginal discs as zones of *DSRF* (*Drosophila* serum response factor) repression (***Montagne et al., 1996***; ***Nussbaumer et al., 2000***).

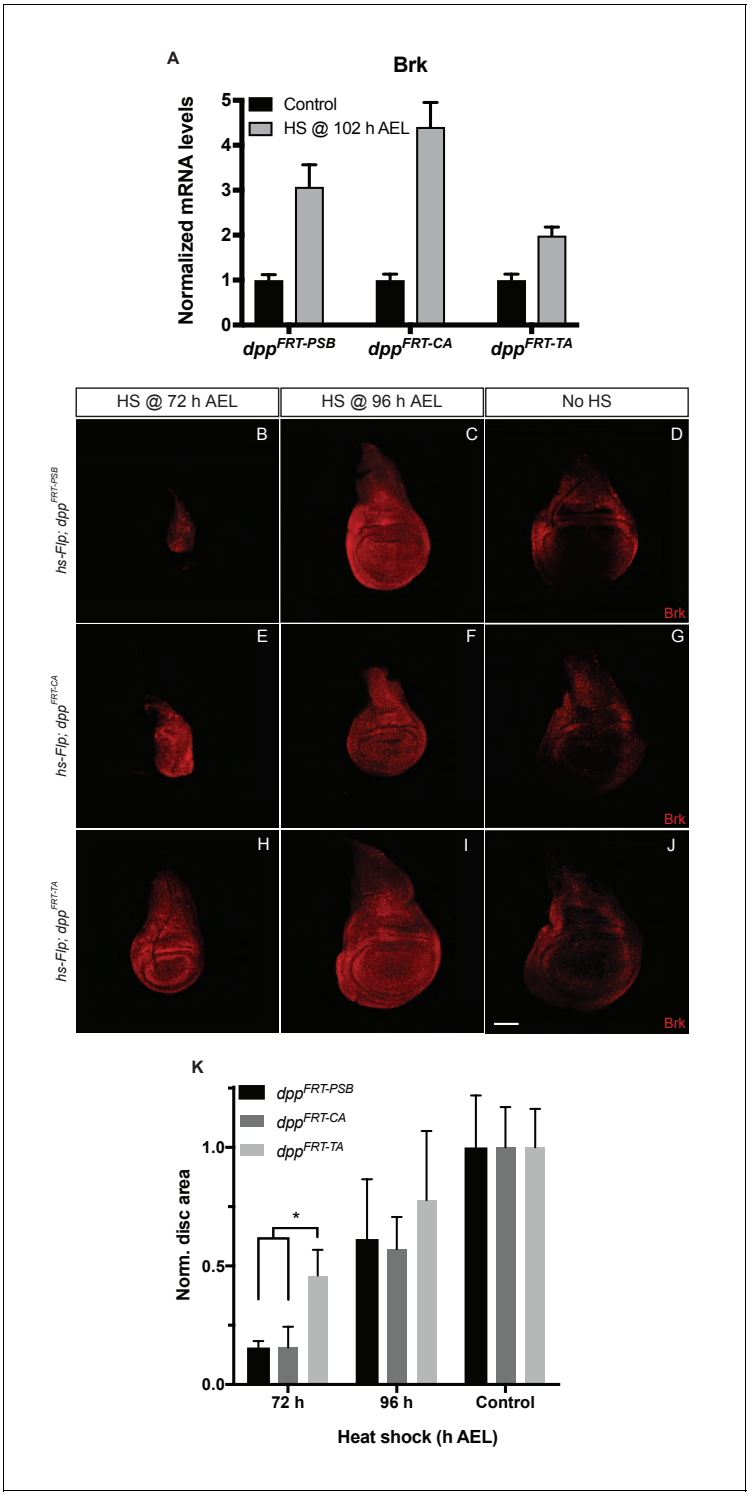

Figure 4. Inactivation efficiency for three conditional alleles of *dpp*. (**A**) Efficiency of inactivation for $dpp^{FRT\text{-}PSB}$, $dpp^{FRT\text{-}CA}$ and $dpp^{FRT\text{-}TA}$ by Flp expressed from *hsp70-Flp* induced at 102 hr AEL. Level of *brinker* mRNA, normalized to that in non-heat-shocked controls, was assessed by qRT-PCR at 120 hr AEL. Each bar shows average mRNA level +/- SEM. A two-way ANOVA test showed statistically different *brinker* expression between $dpp^{FRT\text{-}PSB}$ and $dpp^{FRT\text{-}TA}$ ($p=0.0041$) as well as between $dpp^{FRT\text{-}CA}$ and $dpp^{FRT\text{-}TA}$ ($p<0.0001$). (**B–J**) Imaginal discs of the same genotypes were fixed and stained

Figure 4 continued on next page

Figure 4 continued

with anti-Brinker at 120 hr AEL, following a heat shock at 72 or 96 hr AEL or in the absence of heat shock (control). As can be seen, the 72 hr heat shock did not impair growth as much in $dpp^{FRT\text{-}TA}$ as it did in $dpp^{FRT\text{-}PSB}$ and $dpp^{FRT\text{-}CA}$. (**K**) Quantification of disc surface area (normalized to average surface area of control discs) at 120 hr AEL for the nine conditions shown in panels (**B–J**). Each bar represents data for 10 discs. Asterisk denotes statistical significance, as assessed by a two-way ANOVA test (p=0.029). Scale bar = 50 μm.

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The following source data is available for figure 4:

Source data 1. Primers for qPCR.

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Source data 2. Normalised Brk mRNA levels.

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Source data 3. Total disc area.

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Staining with anti-DSRF showed that the prospective vein pattern was markedly disrupted in 'rescued' discs (***Figure 5G,H***), with only two zones of repressed *DSRF* remaining, one around the D/V boundary, where vein 1 normally forms under the control of Wingless (***Couso et al., 1994***; ***Rulifson and Blair, 1995***), and one around pro-veins 3 and 4, which are specified by Hedgehog in the wild type (***Blair, 2007***). The areas of DSRF repression corresponding to veins 2 and 5 were conspicuously missing. Because some of the 'rescued' larvae survived to adulthood, we were able to further assess, in adult wings, the extent of growth and patterning that uniform Dpp promotes. A majority of these wings appeared to be made entirely of crumpled vein material (***Figure 5O***), which made it difficult to assess size. This phenotype can be explained by the vein-specifying role of Dpp in pupal wings (***Sotillos and de Celis, 2006***). Nevertheless, a minority of 'rescued wings' were remarkably well formed (***Figure 5N***), perhaps because they experienced lower Dpp signalling at the pupal stage, below the threshold for vein specification. In these wings, vein patterning was disrupted, but reproducibly so, with a broad swath of vein tissue forming near the A/P boundary. Crucially, these wings reached a remarkably large size (compare ***Figure 5M and N***). This result suggests that uniform, low level Dpp signalling promotes near-normal growth although this is not adequate for patterning.

Discussion

Dpp behaves as a classic morphogen in wing imaginal discs of *Drosophila*. It is produced from a stripe of cells along the A/P boundary and spreads from there to activate the nested expression of target genes, which in turn position longitudinal veins. In addition to providing patterning information in the prospective wing, Dpp also promotes growth via repression of *brinker*. How graded Dpp signalling leads to homogenous proliferation has been the subject of discussion but until recently, there has been general agreement that the stripe of Dpp is required for growth. This basic tenet was recently challenged with a conditional *dpp* allele that can be inactivated in time and space by Flp (here referred to as $dpp^{FRT\text{-}TA}$). Inactivation in the normal domain of Dpp expression, with Flp driven by a disc-specific *dpp* regulatory element, was reported to have minimal impact on growth (***Akiyama and Gibson, 2015***). The authors suggested that Dpp expressed from a source in the anterior half of the pouch could suffice to sustain growth. Consistent with this suggestion, inactivation of *dpp* throughout the pouch with *nubbin-Gal4 UAS-flp* led to strong growth reduction (***Akiyama and Gibson, 2015***), an observation that we confirmed with our conditional alleles ($dpp^{FRT\text{-}CA}$ and $dpp^{FRT\text{-}PSB}$) and two pouch-specific sources of Flp. However, inactivation of our alleles with dpp^{BLK}*-Gal4 UAS-Flp* (the same source of Flp used by ***Akiyama and Gibson, 2015***) led to a severe impairment in growth (***Figure 3*** and ***Figure 3—figure supplement 3***), in contrast to the finding with $dpp^{FRT\text{-}TA}$. Our analysis of *brinker* expression during the growth period in the various mutant backgrounds allows us to reconcile the apparent discrepancy between our data and those of ***Akiyama and Gibson (2015)***. We suggest that our alleles ($dpp^{FRT\text{-}CA}$ and $dpp^{FRT\text{-}PSB}$) are more readily inactivated than the one generated by ***Akiyama and Gibson (2015)*** ($dpp^{FRT\text{-}TA}$). Thus, in the $dpp^{FRT\text{-}TA}$*; dpp-Gal4 UAS-Flp* genotype, cells expressing Dpp within the stripe would linger long enough to provide sufficient signalling activity for *brinker* repression (***Figure 3H,I***) and hence growth. As time goes on,

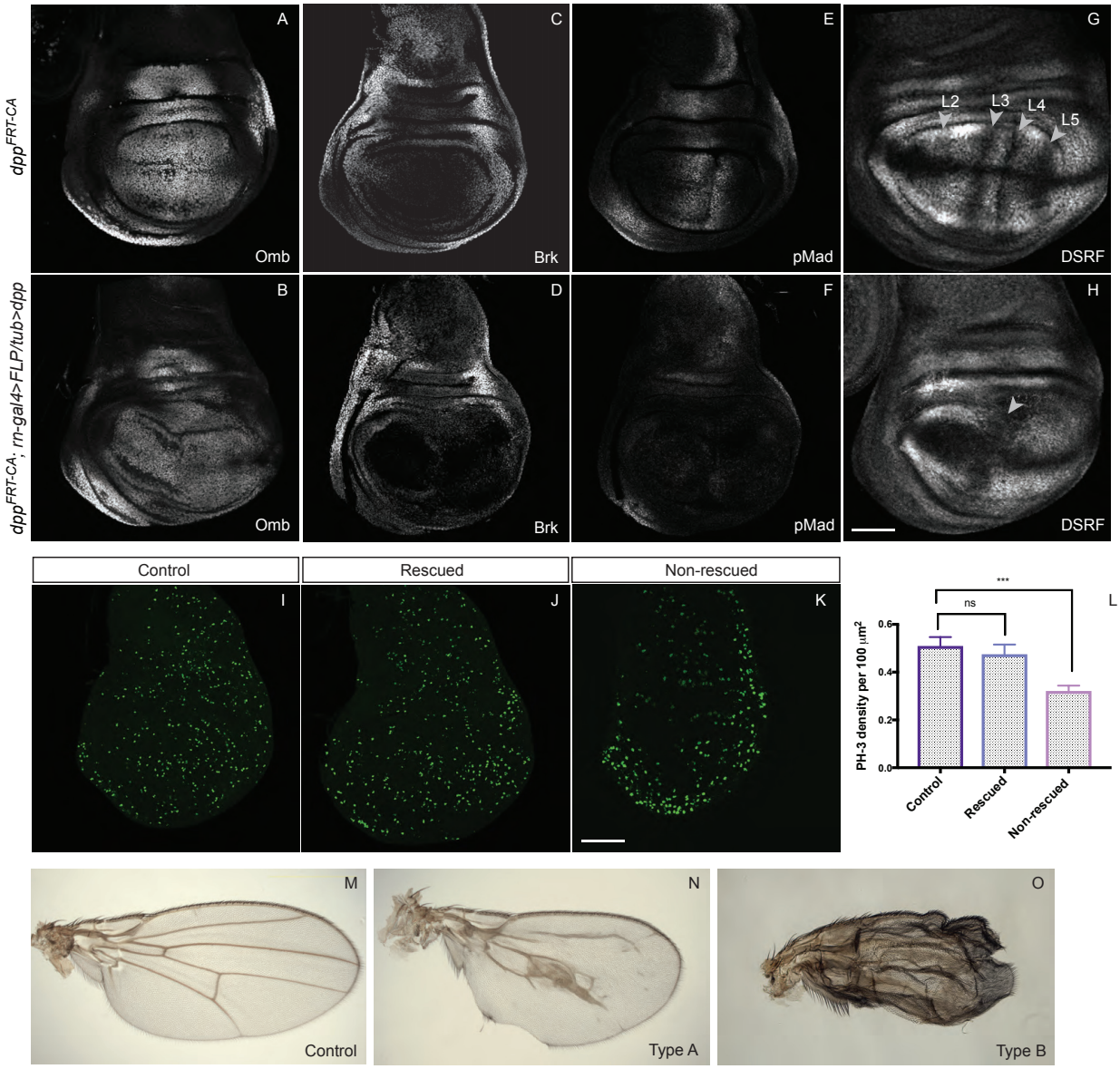

Figure 5. Low level uniform Dpp expression suffices for growth but not patterning. (**A–H**) Comparison of wild-type discs (**A, C, E, G**) to discs lacking endogenous Dpp in the pouch and expressing weak uniform Dpp instead (**B, D, F, H**). Uniform Dpp allows discs to reach a relatively normal size, although with a variably deformed shape (representative examples are shown). Omb is expressed in experimental discs, an indication of active Dpp signaling, but at a relatively lower level than in control discs (samples shown in A and B were stained and imaged under identical conditions). Note also the repression of Brinker and the loss of pMad expression in experimental discs. In contrast to their relatively normal size, experimental discs show abnormal vein patterning, with only two vein territories recognizable instead of the normal five (marked by the absence of DSRF immunoreactivity) (**G, H**). (**I–K**) pH3 immunoreactivity shows that, in control and rescued discs, proliferation is sustained seemingly normally (**I, J**) while proliferation in the pouch of non-rescued discs is depressed (**K**) Quantification show in L is based on 14 rescued discs, 9 controls and 11 unrescued discs. Statistical significance was assessed with a Student's t-test, assuming equal variance and a Gaussian distribution. Mitotic density (pH3 spots/area) was determined for each individual disc using a code written in Fiji (see ***Figure 5—source data 1***). (**M–O**) Wings from the above genotypes. A majority of examined experimental wings (15/20) had excess vein tissue (**O**) while the remainder (5/20) had one central vein around the position of the A/P boundary and another (not visible) along the margin (**I**). Each micrograph is representative of 7–10 discs. Scale bar = 50 µm.

Figure 5 continued on next page

Figure 5 continued

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The following source data is available for figure 5:

Source data 1. PH3 density.

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these lingering cells would progressively undergo excision so that at the end of third instar, no signalling would remain, explaining the widespread derepression of *brinker* seen at the late 120 hr AEL stage (***Akiyama and Gibson, 2015***). Since, with our conditional allele, inactivation of Dpp in the endogenous stripe leads to growth impairment, we conclude that, during normal development, this source of Dpp is needed for growth, although as discussed below, this can be overcome with low-level exogenously expressed Dpp.

How does the Dpp gradient emanating from the Dpp stripe promote growth? Our finding that uniformly expressed Dpp is sufficient for growth suggests that a spatial gradient of signalling is not required. Moreover, the *tubulin* promoter, which was used to drive uniform expression, is expected to be constant over time. Therefore, our result could be taken as evidence against the model that growth depends on continuously rising signalling activity (***Wartlick et al., 2011b***), although it could be argued that even under a condition of uniform expression, signalling could rise if Dpp became more stable over time. Nevertheless, we prefer the simple model whereby, in the prospective wing, Dpp signalling over a threshold would be permissive for growth. The level of this threshold is still to be precisely measured. In the experiment illustrated in ***Figure 5***, growth rescue by uniform Dpp in the pouch correlates with repression of *brinker*, consistent with the growth equalization model (***Schwank et al., 2008***). Although Akiyama and Gibson showed that $dpp^{FRT\text{-}TA}$ dpp^{BLK}*-Gal4 UAS-Flp* discs express *brinker* uniformly at 120 hr AEL (***Akiyama and Gibson, 2015***), as we have shown (***Figure 3H,I***), *brinker* only becomes derepressed in this genotype after growth has occurred. The observations that Dpp expression from the *Tubα1-dpp* transgene (***Figure 4***) or residual Dpp from a few cells within the stripe (as we propose is occurring in the $dpp^{FRT\text{-}TA}$ dpp^{BLK}*-Gal4 UAS-Flp* background), stimulate growth suggest that relatively low level signalling suffices for growth throughout the pouch (i.e. the prospective wing). As we have shown, this level of signalling is below that needed to produce substantial pMad immunoreactivity but higher than that needed to repress *brinker*. Better tools to tune the level of Dpp signalling will be needed to assess the relationship between signalling activity and growth at all stages.

Our results have significantly clarified the spatial requirement of Dpp. As we have shown, Dpp must originate from the pouch for this tissue to grow: in several experimental conditions (***Figure 3B, D***, ***Figure 1—figure supplement 1C–F***, ***Figure 3—figure supplement 3C–D***), Dpp produced outside the pouch could not overcome the absence of Dpp within the pouch. We cannot discriminate at this point whether the boundary between these tissues acts as a barrier to the spread of Dpp or whether these sources of Dpp are too weak to have an impact in the pouch. In any case, these observations confirm our assertion that growth is normally sustained by Dpp produced at the A/P boundary. Dpp signalling above a relatively low threshold is permissive for growth within the pouch throughout wing development. For this activity, the signalling gradient is irrelevant. By contrast, the signalling gradient is essential for patterning as it specifies the domains of *salm* and *omb* expression and thus the positions of veins. Thus, the dual role of Dpp in growth and patterning requires that it is expressed in a stripe. Late inactivation of Dpp impairs patterning, suggesting that the gradient information could be read at the end of the growth period. It remains to be determined how the two processes - growth and patterning - are coordinated to ensure the reproducible formation of the adult wing.

Materials and methods

Drosophila strains

Two conditional *dpp* alleles, illustrated in ***Figure 1B***, were created for this study. In one allele, $dpp^{FRT\text{-}CA}$, the exon encoding mature Dpp was deleted and replaced with the same sequence flanked by FRT and modified so that it would encode two HA tags downstream of the three furin

cleavage sites. For the other allele, $dpp^{FRT\text{-}PSB}$, a portion of the first coding exon including the signal sequence was replaced by a FRT-flanked fragment encoding full-length HA-tagged Dpp (3xHA tag). See **Source data 1** for the full sequence. Both alleles are homozygous viable with no apparent morphological phenotype. Both are fully inactivated by Flp-mediated excision of the FRT cassette. We also generated a reporter allele, $dpp^{FRT\text{-}REP}$, by inserting the DNA fragment shown in **Figure 3—figure supplement 1** in the *attP* site of the deletion allele used to generate $dpp^{FRT\text{-}CA}$ (see **Figure 1B**). In this construct, CD8-GFP coding sequences are located downstream of an HA-Dpp excisable cassette. See **Source data 1** for the full sequence. The dpp^{FO} allele (**Akiyama and Gibson, 2015**), referred to here as $dpp^{FRT\text{-}TA}$ was obtained from Matt Gibson (Stower's Institute). Tubα1-FRT-f^{+}-FRT-Dpp was described previously (**Zecca et al., 1995**). The other strains used for this study were obtained from the Bloomington stock centre. They include *rotund-Gal4 (rn-Gal4), nubbin-Gal4 (nub-Gal4), tubulin-Gal80^{ts}(II) (tub- Gal80^{ts}), UAS-Flp (X), hs-Flp (X) and hs-Flp (III).*

PCR analysis of genomic DNA

For Splinkerette PCR, DNA from single flies was isolated and digested with BglII. Afterwards, it was amplified following the Splinkerette PCR protocol for *Drosophila melanogaster* (**Potter and Luo, 2010**). Three dpp^{BLK}-*Gal4* lines (which were kept in three labs for extended time) were analysed: $dpp^{BLK\text{-}TA}$-*Gal4* (**Akiyama and Gibson, 2015**), $dpp^{BLK\text{-}CA}$-*Gal4* (kept in London) and $dpp^{BLK\text{-}PSB}$-*Gal4* (kept in Zürich). The following primers were used: SPLNK#1 + 5'SPLNK#1-GAWB for the first PCR round and SPLNK#2 + 5'SPLNK#2-GAWB for the second PCR round (see **Figure 4—source data 1** for primer sequences). The PCR products were isolated on a 2% agarose gel and sequenced with the primer 5'SPLNK-GAWB-SEQ. The size of the fragment differed for the three strains, probably because of polymorphism that accumulated during maintenance of the stocks. However, sequencing of the fragment showed that in all three cases, the insertion sites were identical, in the 5'UTR of CG6896 (MYPT-75D).

qRT-PCR

Third instar larvae were heat shocked for 30 min at 102 hr AEL and wing discs were dissected in PBS at 120 hr AEL, before being transferred to PBS-Tween 20. Samples were spun down, and the pellets were snap-frozen in liquid nitrogen, stored at −80°C or processed immediately. RNA from the dissected discs was extracted with the Macherey-Nagel NucleoSpin RNA isolation kit, and cDNA was obtained with the Roche Transcriptor high fidelity cDNA synthesis kit. Quantitative PCR was performed in triplicates using the MESA Green qPCR Mastermix Plus for SYBR assay. All measurements were normalized to *actin-5C*, *alpha-tubulin* and *TATA box binding protein* mRNA levels. See **Figure 4—source data 1** for primer squences.

Imaging

Imaginal discs were fixed in 4% paraformaldehyde for approximately 30 min before immunofluorescence staining. The following antibodies were used: α-Brinker (Aurelio Telemann, EMBL; 1/500), α-Brinker (Hillary Ashe, University of Manchester; 1/500); α-HA (Cell Signalling; 1/3000 or 1/500), α-Hth (Richard Mann, Columbia University; 1/500), α- Phospho-Histone H3 (Abcam; [HTA28] phospho S28; 1/500), α− Phospho-Smad1/5 (Cell Signalling; 41D10 #9516; 1/100)

α-DSRF (Active Motif; Cat 39093 Lot 03504001; 1/500), α-Omb (Gert Pflugfelder, University of Mainz; 1/500), and Alexa-conjugated secondary antibodies (Thermo Scientific Waltham, MA; 1/500). Images were acquired either with a Zeiss LSM710 or a Leica SP5 confocal microscope.

Data analysis

Every experiment was repeated at least once. All data were analysed using Fiji (ImageJ) and GraphPad Prism. Error bars denote standard deviation (SD) unless stated otherwise, and the statistical tests used to evaluate significance are described in the figure legends. Statistical significance is denoted as follows: ns: $p>0.05$, *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$.

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PSB, RZ, Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review and editing; CA, Conceptualization, Formal analysis, Supervision, Investigation, Methodology, Writing—original draft, Writing—review and editing; KB, Conceptualization, Resources, Formal analysis, Supervision, Funding acquisition, Investigation, Writing—original draft; J-PV, Conceptualization, Resources, Supervision, Funding acquisition, Methodology, Writing—original draft, Project administration, Writing—review and editing

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Additional files

Supplementary files

• Source data 1. Allele sequences.
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Supplementary figures

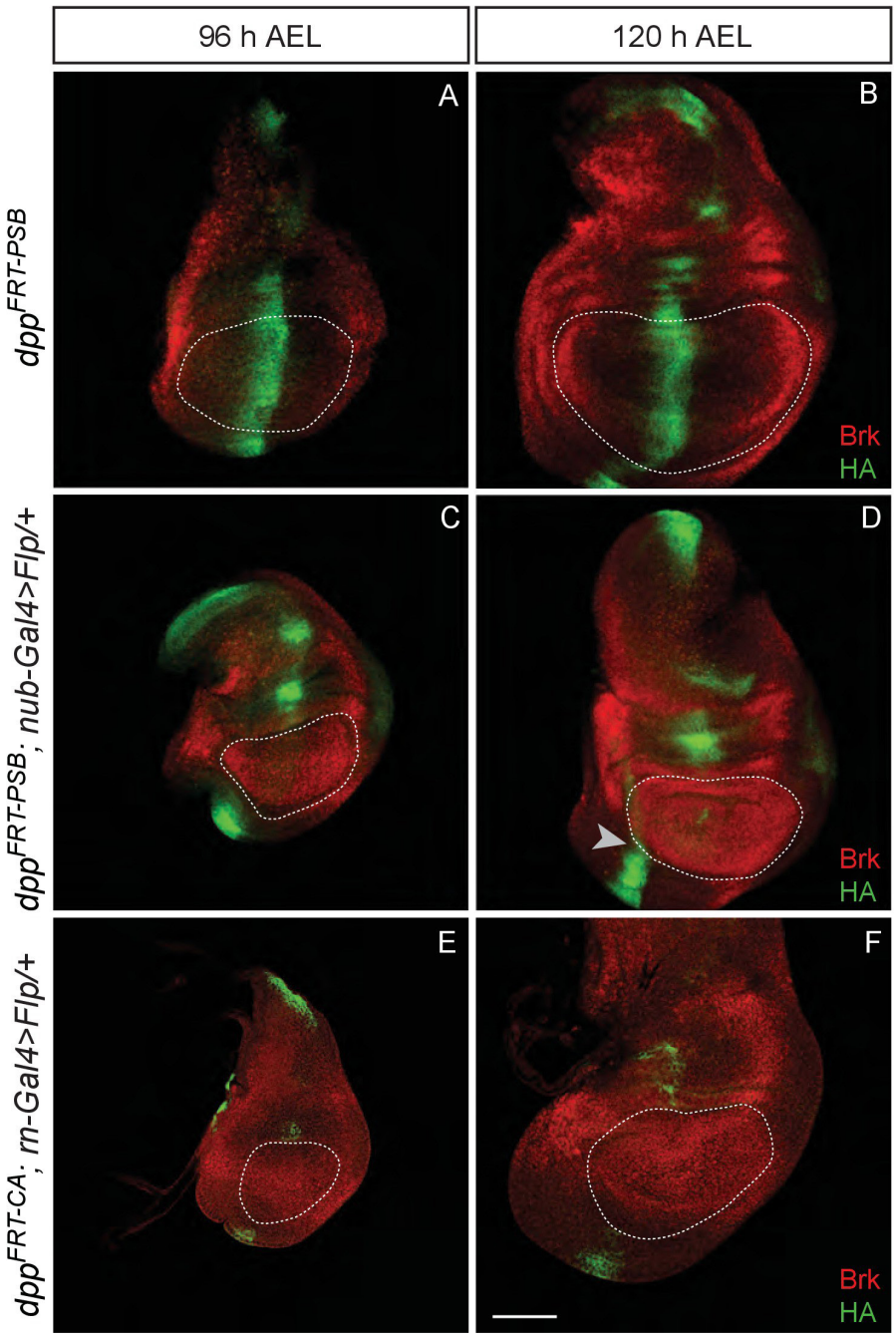

Fig. 1 Suppl. 1. Inactivation of Dpp specifically in the pouch.

(A–B) Expression of HA-Dpp and Brinker in the unexcised $dpp^{FRT\text{-}PSB}$ allele (normal Dpp activity). Note the repression of brinker expression on either side of the central stripe of Dpp. (C–D) Pouch-specific inactivation of Dpp expression from $dpp^{FRT\text{-}PSB}$ by nubbin-gal4 UAS-Flp. Most HA immunoreactivity has disappeared at 96 hr AEL, although some is still detectable at 72 hr AEL (not shown). As Dpp disappears, brinker becomes derepressed. (E–F). Pouch-specific inactivation of Dpp expression from $dpp^{FRT\text{-}CA}$ by rotund-gal4 UAS-Flp. As with nubbin-Gal4, Dpp is no longer detectable in the pouch from 96 hr AEL. On all panels, the edge of the pouch (marked with a white dotted line) was estimated from tissue folds that could be observed in the DAPI channel (not shown). Scale bar = 50 µm.

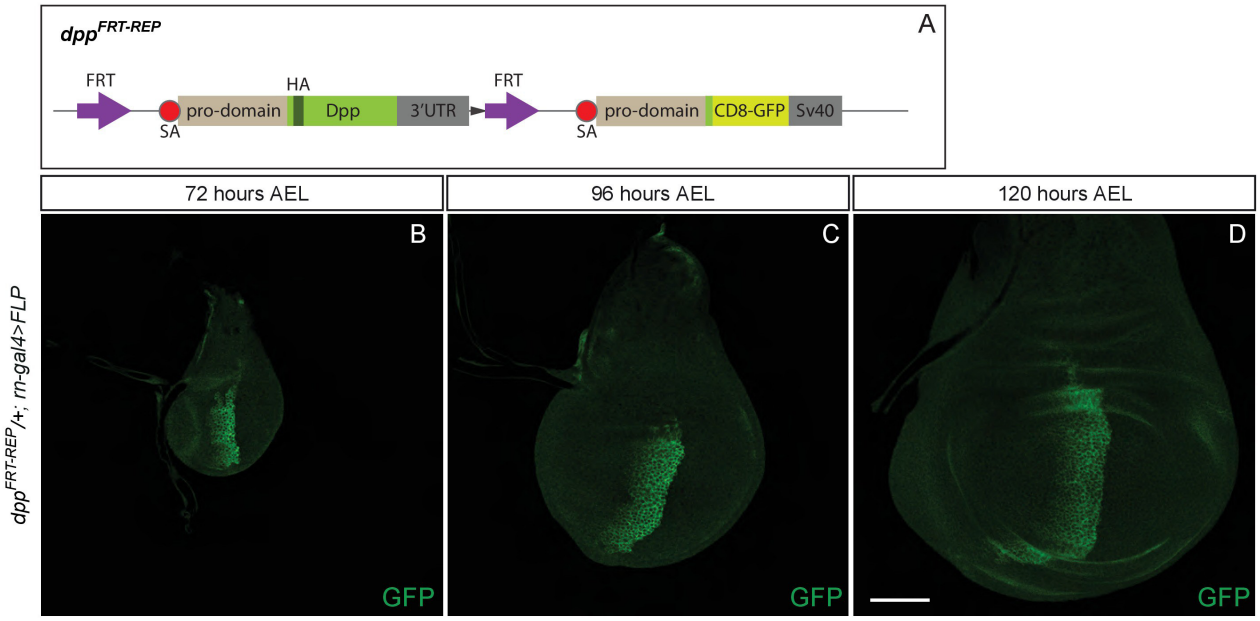

Fig. 3 Suppl. 1. A reporter inserted at the locus shows that dpp expression is confined to the stripe along the A/P boundary.

(A) Diagram describing the $dpp^{FRT\text{-}REP}$ allele, designed to act as a reporter following excision of the FRT-flanked HA-Dpp-containing cassette. B-D. Expression of CD8-GFP from $dpp^{FRT\text{-}REP}$ within the pouch at 72, 96, and 120 hr AEL. Expression is only seen in the stripe. Scalebar = 50 μM

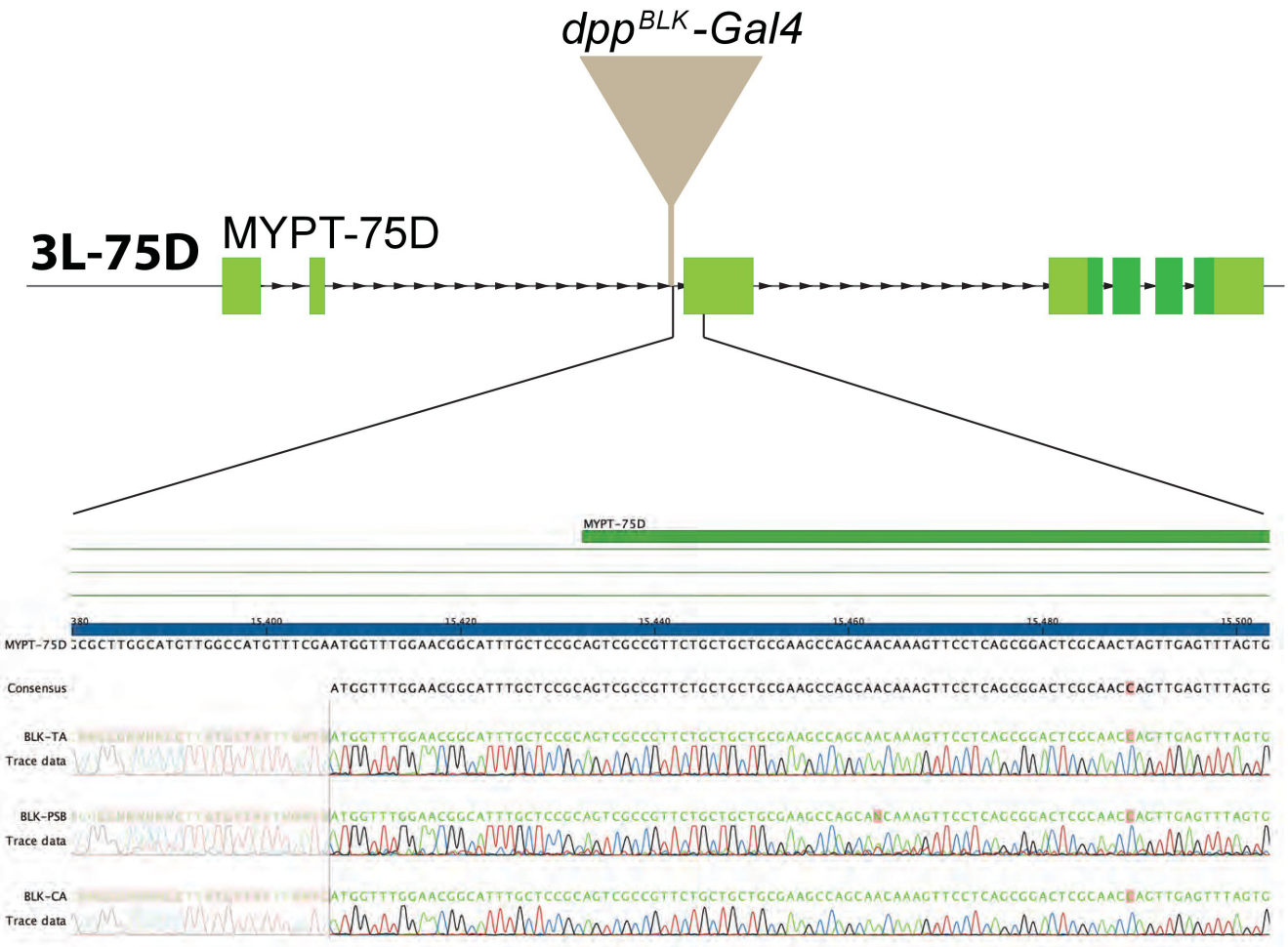

Fig. 3 Suppl. 2. Comparison of various dpp-Gal4 strains.

Three dpp^{BLK}-Gal4 lines (kept separately in three laboratories) were characterised molecularly, as described in Materials and methods. DNA sequencing of the genomic region flanking the transgene shows that the insertion site is the same for all three strains.

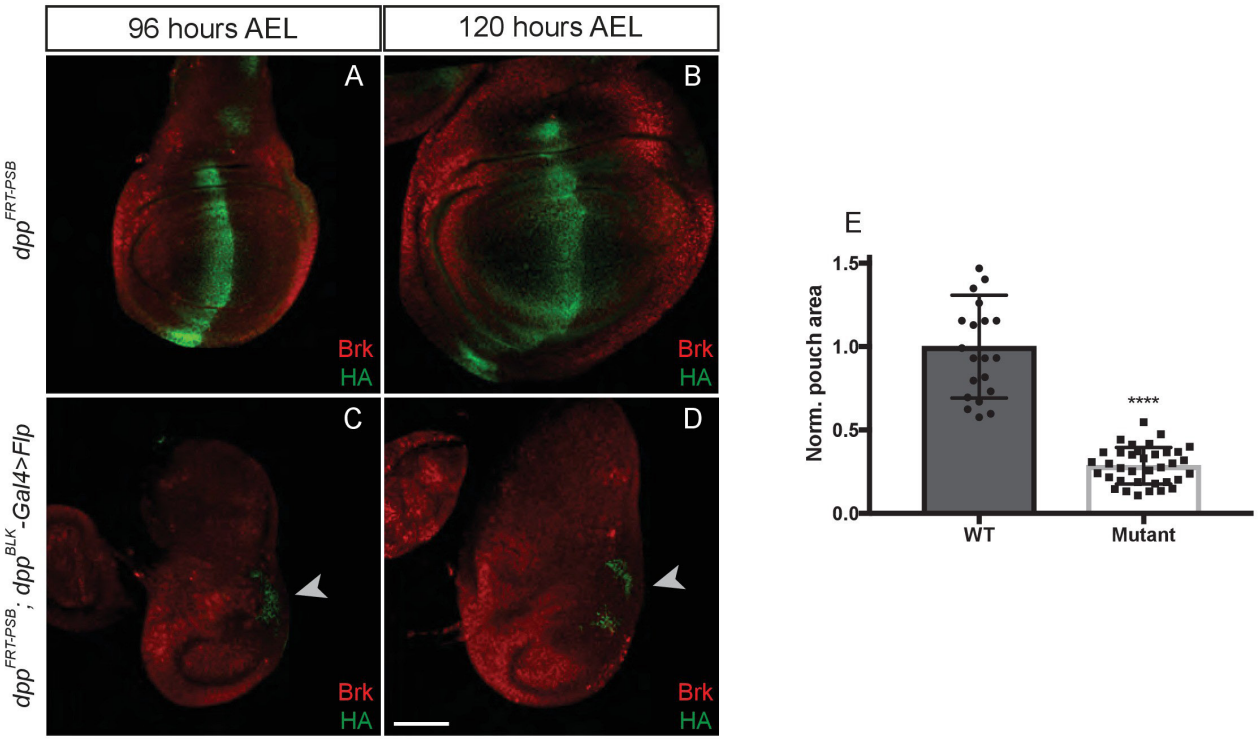

Fig. 3 Suppl. 3. Inactivation of dppFRT-PSB in the domain of dpp expression abolishes growth.

(A–D) Inactivation of dpp$^{FRT\text{-}PSB}$ in the normal Dpp expression domain with dpp^{BLK}-Gal4 UAS-Flp leads to derepression of ***brinker*** and growth defects. Note the repression of brinker at the posterior end of the disc (arrowheads in C and D). (E) Quantification of the pouch area in control and experimental discs. Each dot/square represents a disc. The statistical difference ($p<0.0001$) was calculated with a t-test, assuming equal variance and a Gaussian distribution. Scale bar = 50 µm.

Chapter 4. Visualizing the establishment of the Dpp gradient in *Drosophila* wing imaginal discs

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Abstract

In the wing imaginal disc, Dpp is produced at the anterior-posterior boundary, from where it diffuses to create the signaling gradient that regulates growth and patterning during development. The gradient is tightly regulated to be able to scale with the growing tissue during development. The mechanism by which the gradient is established and scaled is not yet fully understood. Previous work on the Dpp gradient was based on the overexpression of a GFP-tagged Dpp transgene, using the Gal4>UAS system. Despite its versatility, Dpp overexpression has a drawback, as both the intracellular and extracellular Dpp pools are much higher than under endogenous conditions. In this study, we devised a new strategy, where we replaced the endogenous Dpp with a convertible tandem construct that expresses one of two differently tagged Dpp variants. This construct was integrated into the *dpp* locus. Upon deletion of one of the copies in the tandem from the genome, we can track and estimate protein production, diffusion and degradation, effectively measuring the parameters to model the Dpp gradient formation in a accurate manner.

Introduction

Morphogens are small signaling molecules that coordinate the two critical developmental processes: growth and patterning. They are produced from a localized source and secreted into the extracellular space. Once secreted, they move or are transported through the tissue, creating a concentration gradient. Cells closer to the source will be exposed to a higher morphogen concentration, and will express a subset of high threshold genes. Cells farther from the source will receive less or no morphogen and thus expressing different sets of genes. The concentration-dependent gene expression was first postulated by Lewis Wolpert in a model known as the French Flag (Wolpert, 1969). In this model different thresholds of morphogen concentration will determine the fate of different cell groups. Each of these cell groups expresses a distinct subset of genes and they will end up differentiating into the various cell populations that compose the adult tissue. This model has been supported over the years with solid evidence (Affolter and Basler, 2007; Hamaratoglu et al., 2009; Rogulja and Irvine, 2005; Schwank and Basler, 2010; Wartlick et al., 2011).

The morphogen gradient shape and length will determine the fates of the different subsets of cells. Therefore, morphogen movement has to be tightly regulated (Müller et al., 2013). To study how morphogens can spread from its source and create a stable long range gradient, the movement of Decapentaplegic (Dpp) in the *Drosophila* wing disc has been very widely employed (Entchev et al., 2000; Nellen et al., 1996; Teleman and Cohen, 2000). This is due to the many advantages that the development of the Drosophila wing offers: 1) the development of the fruit fly wings is characterized in detail, 2) the structure of the adult wing is relatively simple and has canonically localized substructures, 3) the wing precursor develops in just three days and exhibits an incredible growth rate that renders a structure of around 50,000 cells from a precursor of barely 50 cells (Held, 2005).

How does Dpp move from its source to establish the gradient? Five different hypotheses have been proposed to describe how a pool of Dpp morphogen reaches the steady state, where a stable long-range gradient establishes the pattern of the tissue while driving homogeneous growth throughout it. These hypotheses are represented in Fig. 1 and reviewed in (Müller et al., 2013; Restrepo et al., 2014). The simplest model attributes the establishment of the gradient to free extracellular diffusion (Fig. 1A), where the morphogen travels through the extracellular region until it reaches the receptors, activating the downstream signal (Zhou et al., 2012). However, the measured diffusion coefficient appears to be too slow for free diffusion (Entchev et al, 2000; Schwank et al, 2011). Therefore, instead of moving freely, the second hypothesis postulates that the gradient is achieved via restricted extracellular diffusion (Fig. 1B), where either Dpp receptors or glypicans, a family of heparan sulfate proteoglycans (Filmus and Selleck, 2001), restrict the ability of the morphogen to travel (Schwank et al., 2011). The third model, adds another layer of interaction to the restricted diffusion: an additional molecule, called expander (or facilitator), interacts with the glypicans to increase Dpp diffusion. This model is known as facilitated diffusion, or expansion-repression. According to this hypothesis, the hindrance to the morphogen diffusion is reduced by the expander. This expander is produced from the lateral regions of the discs and diffuses to help Dpp unbind from the glypicans (Fig. 1C), allowing it to travel farther. In the case of Dpp diffusion in the wing disc, Pentagone, a small molecule produced in an inverted gradient compared to the one of Dpp, has been found to act as expander and help the Dpp gradient to expand and scale

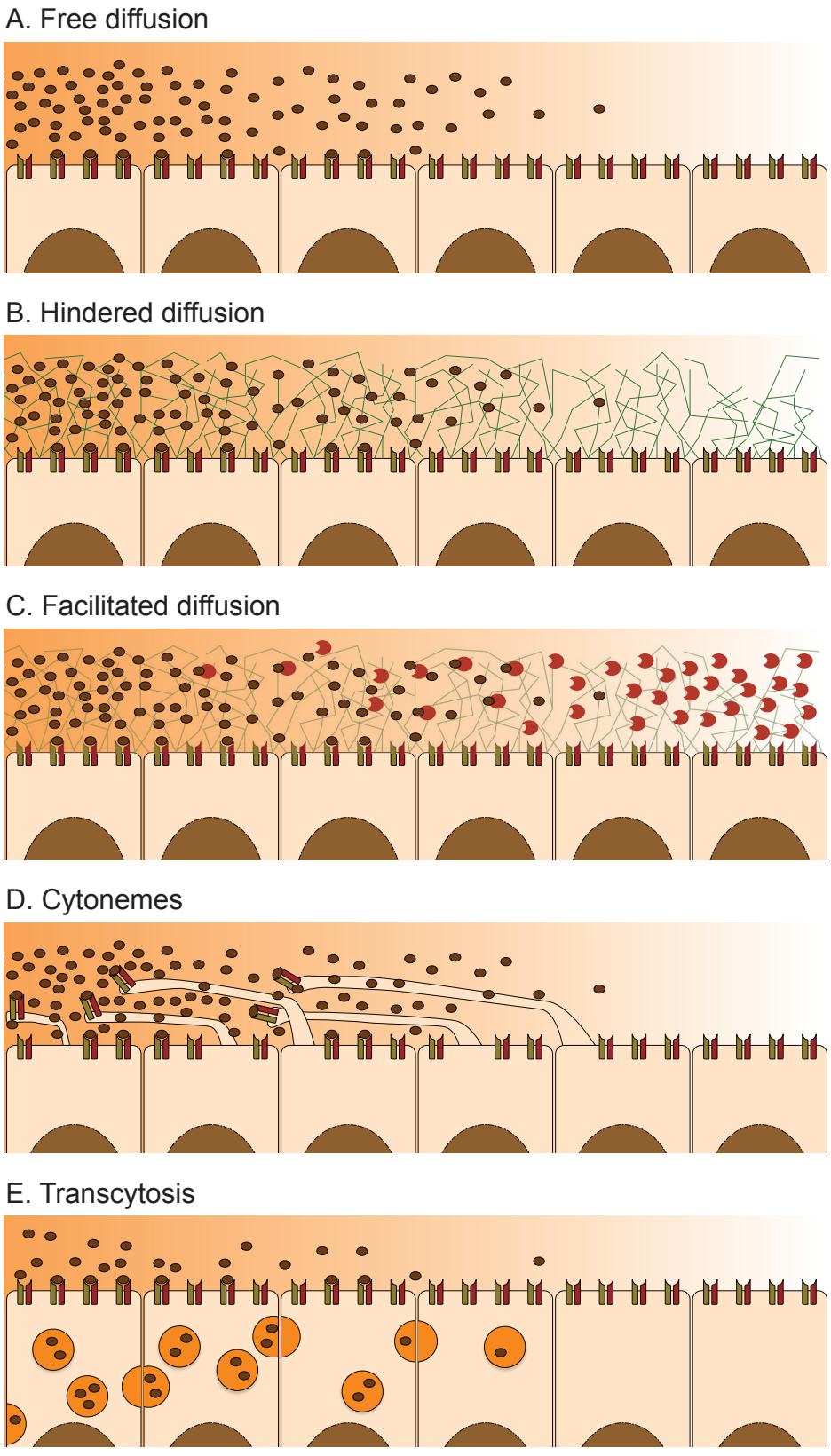

Figure 1. Models for Dpp diffusion

A) Free extracellular diffusion of Dpp. B) Restricted extracellular diffusion, where Dpp is bound to glypicans and receptors, hindering its diffusion. C) Facilitated diffusion, or expansion-repression mechanism. Pentagon (the expander) interacts with glypicans, facilitating the diffusion of Dpp. D) Cytonemes protrude from the lateral regions of the disc and reach Dpp at the source. Dpp bound to the receptors is pulled farther from the source, extending the gradient. E) Transcytosis of Dpp, where it is passed from cell to cell by succesive rounds of exo- and endocytosis.

with wing size (Hamaratoglu et al., 2011; Vuilleumier et al., 2010; Ben-Zvi et al., 2011). The remaining two hypotheses rely on active Dpp transport, carried out by the cells. In the fourth model, instead of an expander to help the morphogen diffuse, Dpp is captured by long actin-based cellular

protrusions known as cytonemes, which extend from the lateral regions of the wing disc towards the center (Fig. 1D). The cytonemes contain Dpp receptors in their surface, which reach the morphogen directly at its source. Once these receptors bind Dpp, the morphogen is pulled from the source, effectively extending the gradient towards the lateral regions (Hsiung et al., 2005; Ramirez-Weber and Kornberg, 1999; Roy et al., 2014). The fifth hypothesis postulates that morphogens are transferred from cell to cell in a series of rounds of dynamin-mediated exo- and endocytosis, but not freely-diffusing. This process is known as transcytosis (Fig. 1E) (Entchev et al., 2000; Kicheva et al., 2007; Wartlick et al., 2011b).

Despite decades of continuous research on the topic, there is still no consensus in regards on how the Dpp gradient is established. One of the main caveats of the research performed on the Dpp morphogenetic gradient is the lack of a proper antibody to detect the secreted protein. Therefore, all current models are based on the detection of a fluorescently-tagged Dpp transgene, expressed under its own regulatory region by the Gal4>UAS binary system. Although dpp-Gal4 drives UAS-mediated expression at the Dpp endogenous expressing region, the Gal4 system leads to overexpression, and therefore it does not accurately mimic the endogenous Dpp expression levels. In fact, flies where the only source of Dpp is a Gal4>UAS-driven transgene show wings with an abnormal wing size and defects in patterning compared to wild-type wings (Teleman and Cohen, 2000; Wartlick et al., 2011b). In a system as sensitive and regulated as the establishment of a morphogenetic gradient, overexpressing the signaling molecule can saturate the regulatory partners and lead to ambiguous or wrong interpretations. Therefore, it is important to devise a different approach to assay a stable endogenous morphogen gradient.

We modified the endogenous *dpp* locus to replace the coding sequence by an engineered dpp tandem, where each of the two copies is fused to a different protein tag. The first copy is flanked by FRT sequences, allowing us to cut it out from the genome by inducing Flp-mediated recombination (Golic and Lindquist, 1989). This approach allows tracking the secreted protein after it is no longer produced, as well as tracking the newly produced Dpp, to study morphogen diffusion while there is a steady state gradient. Using this model, we measured the levels of Dpp in the wing disc and how the gradient behaves over time. Our data suggests that the Dpp gradient is created by hindered diffusion, where Dpp is secreted and trapped in the

extracellular region over a long period of time, keeping a stable gradient of morphogen. In addition, we believe that our system will enable a more in depth investigation of the establishment of the Dpp gradient and the interaction with extracellular proteins to regulate and scale the gradient to achieve proper Dpp signaling throughout development.

Experimental Procedures

Drosophila strains

The following fly strains were generated and used in the experiments: FRT-3xHA::Dpp-FRT-3xOLLAS::Dpp ($Dpp^{HA>OLLAS}$), FRT-eGFP::Dpp-FRT-tagRFPt::Dpp ($Dpp^{GFP>tagRFPt}$), UAS-3xHA::Dpp, UAS-3xV5::Dpp, UAS-3xOLLAS::Dpp, UAS-eGFP::Dpp, UAS-TagRFPt::Dpp, UAS-mKate2::Dpp and UAS-mEOS2::Dpp. In addition, we used the following fly stocks: hs-Flp (X), dppBLK-Gal4, ap-Gal4, UAS-Flp (X), tub-Gal80ts (II), VDRC lines (Dally, Dlp, Tkv, Punt, Pent, Shi), nos-Cas9 (Port et al. 2014), and attP-86Fb (Bischof et al., 2007).

Cloning procedures

The dpp locus was targeted with two CRISPR gRNAs (see Appendix 1: Primers and DNA sequences) and a single stranded donor DNA to replace part of exon 5 by an attP sequence, generating the dpp^{attP} allele.

Fluorescent proteins and tag sequences were optimized for codon usage in Drosophila and flanked by NotI restriction sites. They were inserted into the dpp CDS, at the N-ter of the mature peptide sequence. All transgenes were first cloned into a pUASTB vector (Groth et al., 2004) to assess the efficiency of all fusion proteins. Afterwards, selected dpp transgenes were cloned into the RIVFRT-MCS-FRT-MCS3 vector (Baena-Lopez et al., 2013) and integrated into dpp^{attP}, generating the $Dpp^{GFP>TagRFPt}$ and $Dpp^{HA>OLLAS}$ strains.

Immunostaining

Imaginal discs were dissected in Ringer solution and fixed with Formaldehyde 4% + 0.1% Triton X-100 for 30 min. After washing 3 times

with PBT/NA-azide 0.1% discs were blocked with 2% HINGS and stained overnight with primary antibodies. Secondary antibody staining was performed after washing 3 times with PBT/Na-azide 0.1%, with Thermo Scientific Alexa antibodies, at 1:500. Secondary antibodies were washed 3 times with PBT/Na-azide and discs were mounted in Vectashield®. Primary antibodies and concentrations: Cell Signaling rabbit α-HA, 1:3000, Thermo Scientific rat α-OLLAS, 1:100, Promega rat α-V5, 1:500, guinea pig α-Brk, 1:500 (Doumpas et al., 2013).

Extracellular Dpp staining

The protocol for extracellular immunostaining was adapted from (Strigini and Cohen, 2000). Discs were dissected in Schneider's solution and stained with primary antibodies 1 h at 4°C. Discs were then washed 3 times with PBS and fixed 20 min with 4% Formaldehyde without detergent. For subsequent intracellular staining, discs were fixed for another 20 min with 4% Formaldehyde + 0.1% Triton X-100 and stained with the conventional immunostaining described above. The following primary antibodies were used for extracellular immunostaining: Cell Signaling rabbit α-HA, 1:300 and Thermo Scientific rat α-OLLAS, 1:3.

Data acquisition

The areas of the wing disc expressing Dpp were calculated with Fiji. Data normalization, mean and SD/SEM were calculated with Graphpad Prism.

Fluorescent intensity profiles were acquired with Fiji and exported to Matlab for data analysis. Intensity graphs were normalized and averaged in Matlab. To calculate extracellular Dpp pool, the areas of the intensity profile were measured in Matlab. Mean and SD/SEM were calculated with Graphpad Prism.

Results

A tandem Dpp construct to assess morphogen degradation and production

In order to knock out dpp to determine degradation rates without altering the main signaling, we devised a dual system with traceable proteins expressed under the endogenous locus to avoid Dpp signal saturation. To this end, we replaced the endogenous dpp ORF by a transgene carrying two copies of dpp, each fused to a different tag to allow the localization of the mature peptide (Fig. 2a). The first copy of the tandem is flanked by FRT sequences, enabling us to cleave it from the genome to track the degradation of the existing protein pool once the genomic copy is not present anymore.

Since our tandem is expressed at endogenous levels, we predicted that the Dpp signal would be much lower than the one observed in previous studies. For that reason, we tested different protein tags and fluorescent reporters to select the combination of the two fusion proteins that would produce the highest signal. We assayed four fluorescent proteins (eGFP, TagRFPt, mKate2, mEOS2) and three peptide-based tags (3xHA, 3xV5 and 3xOLLAS), based on their good performance (Shaner et al., 2005). All transgenes were fused to the *dpp* CDS flanked by its regulatory 5' and 3' UTR. The transgenes were cloned downstream of the UAS promoting element, and inserted into the third chromosome. Expression of the transgenes was driven via the $dpp^{BLK\text{-}Gal4}$ driver line, used in former studies of the Dpp gradient. We drove the expression of all transgenes for 24 hrs before dissections (Fig. 2). After assessing the intensity and resemblance to the Dpp gradient for all the transgenes, we combined the two best fluorescent proteins and the two best peptide tags into two different tandems. From the fluorescent proteins, eGFP and TagFRP-t performed better than mKate2 and mEOS2 (Fig. 2E-H), and from the peptide tags, HA and OLLAS produced good staining, while V5 was not detectable (Fig. 2B-D). In all cases, the expression levels were lower than those shown in the literature, likely because all transgenes carried the endogenous Dpp 5' and 3' UTR.

To integrate the chosen transgenic tandems in the genome, we used the CRISPR/Cas9 system. We followed a strategy established in our group to replace dpp by a tagged, excisable transgenic version of the gene (Sanchez

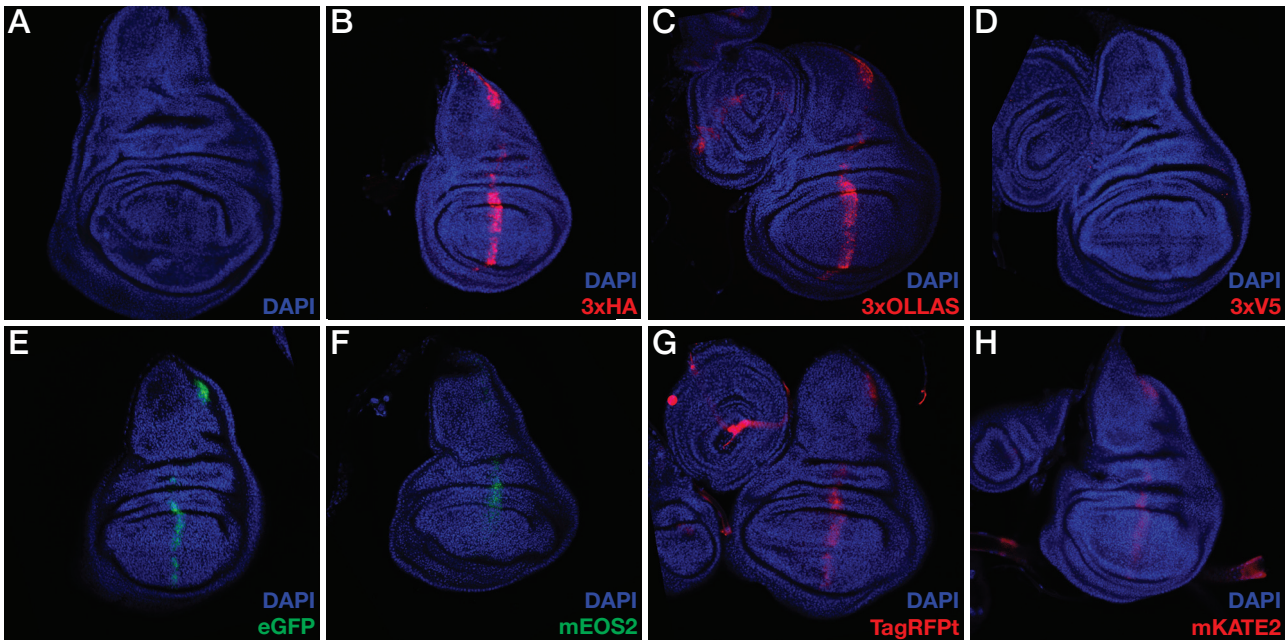

Figure 2. Assessing the activity of the different tagged Dpp transgenes

All tagged Dpp constructs were expressed with the Gal4>UAS system, driving its expression in the endogenous region via dpp^{BLK}-Gal4. A) Control discs expressing UAS-dpp without any tag. B-H) Expression pattern of the different Dpp fusion proteins generated. All transgenes were expressed for 24 hrs before dissection to assess the effectivity of the protein expression and maturation in a short timeframe. B) UAS-3xHA::Dpp. C) UAS-3xOLLAS::Dpp. D) UAS-3xV5::-Dpp. No V5 expression could be detected. E) UAS-eGFP::Dpp. F) UAS-mEOS2::Dpp. G) UAS-tagRFPt::Dpp. H) UAS-mKate2::Dpp.

Bosch et al., 2017) to integrate the tandem inside the dpp genomic region, controlled by all endogenous regulatory elements of *dpp* (Fig 3A). We named the transgenic flies $Dpp^{GFP>tagRFPt}$ or $Dpp^{HA>OLLAS}$, depending on the tandem they carried. When analyzing the expression of the fluorescent protein tandem ($Dpp^{GFP>tagRFPt}$), we observed that the levels of Dpp were too low to efficiently measure intracellular or extracellular Dpp pools from the fluorescent signal (Fig 3B-C). The protein tags, however, yielded much better results (Fig. 3D-E). By using the peptide tags we could also differentiate extracellular and intracellular Dpp pools via different immunostaining assays (see methods). Therefore, we focused on using the epitope tags for our subsequent assays. We will refer to the products of that tandem as Dpp^{HA} and Dpp^{OLLAS}.

Dpp expression pattern produced from both copies of the tandem was checked by immunostaining (Fig. 3D-E). Both Dpp^{HA} and Dpp^{OLLAS} were functional as revealed by downstream *brk* expression (Fig 3E-F) and adult wing morphogenesis (Fig 3F-G). The extracellular fraction of the protein (exDpp) was also detectable by immunostaining (Fig 5A), although at much lower levels than prior studies using the dpp-Gal4>UAS-GFP::dpp

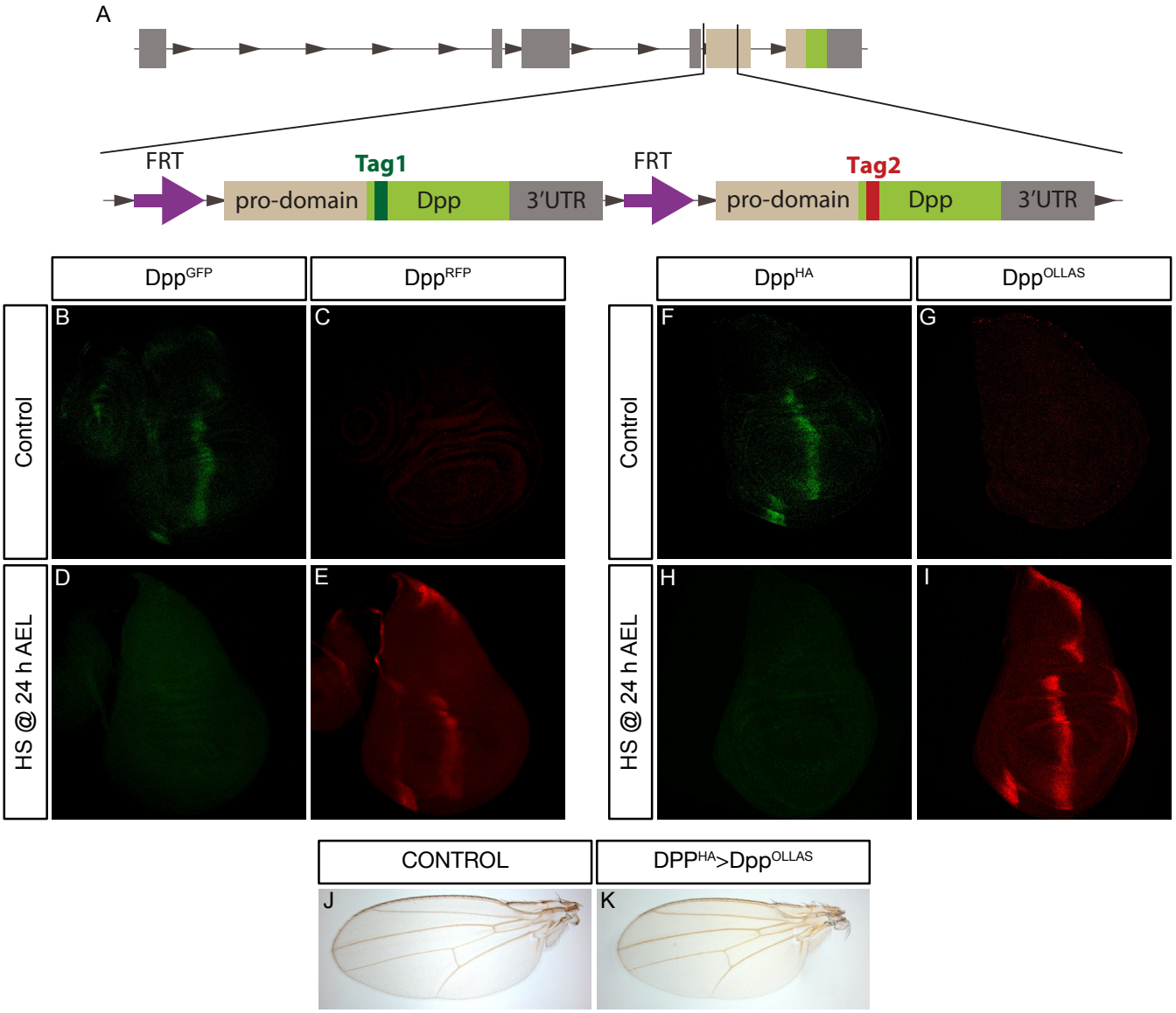

Figure 3. Functional analysis of the Dpp tandem

A) Schematic of the Dpp tandem inserted into the *dpp* locus. Part of the first coding exon was replaced by the tandem. Tandem expression is regulated by the endogenous 5'UTR and each of the tandem copies carries the *dpp* 3'UTR. B-E) Expression of the Dpp$^{GFP>tagRFPt}$ tandem. B-C) GFP (B) and tagRFPt (C) fluorescence before activation of the Flp recombinase. D-E) GFP (D) and RFP (E) expression after expressing the Flp recombinase for 48 hrs. F-I) Expression of the DppHA>OLLAS tandem. F-G) Staining against HA (F) and OLLAS (G) before induction of the Flp recombinase. H-I) Staining against HA (H) and OLLAS (I) 48 hrs after induction of the Flp recombinase. J-K) Adult wings of control (J) and homozygous Dpp$^{HA>OLLAS}$ flies (K).

(Schwank et al., 2011; Wartlick et al., 2011b). This is not surprising, considering the fact that Dpp is expressed at endogenous levels in our system. This finding already suggests that former studies in the field might have saturated the extracellular space with very high concentrations of morphogen, potentially leading to misinterpretation of results.

In conclusion, we have generated a viable, fully functional tandem system to study the Dpp morphogenetic gradient in the *Drosophila* wing ima-

ginal disc. This tandem is expressed at endogenous levels and keeps the protein functionality.

Dpp degradation is a slow process

Former studies of the Dpp gradient argued that free diffusion would require fast recycling of morphogens, whereas slow hindered diffusion needs also reduced protein internalisation and degradation (Zhou et al., 2012). In order to discriminate whether Dpp diffuses freely or is hindered, we measured the speed at which Dpp^{HA} gets degraded after the gradient has achieved the steady-state. We combined our Dpp tandem with a genetic background where we could cleave the first tandem copy (dpp^{HA}) only in the dorsal compartment. By using $Gal80^{ts}$, we controlled the temporal induction of the cleavage by shifting the larvae to 29ºC. This provides us with controllable *dpp* cleavage timing and allows examining the temporal dynamics of the Dpp gradient. As the ventral compartment remains unaltered with this approach, we used it as an internal control to measure Dpp^{HA} levels at the steady state (Fig. 4B).

To measure Dpp degradation, we need to ensure that no more protein is being secreted to the extracellular pool from the remaining intracellular pool after cleavage. Therefore, we first measured the speed at which Dpp^{HA} stops being produced after cleavage of the genomic dpp copy. This timeframe includes the time the FLP recombinase takes to be expressed, cleaves Dpp from the genome, and most of the mRNA from the first tandem copy is degraded. To measure it, we stained intracellular Dpp and quantified when its presence is negligible. We measured the dorsal Dpp^{HA} and $Dpp^{O\text{-}LLAS}$ levels every 3 hrs after shifting larvae to 29ºC. We estimated the intracellular Dpp production by two separate approaches. First, we measured the area of cells expressing each of the Dpp copies, measuring when the area of cells with Dpp^{HA} is negligible (Fig. 4B). Representative discs of the shift process are shown in Fig. 4B-E, and the areas extracted from these discs in Fig. 4B'-E'. The second approach consisted of averaging the intensity of the fluorescent signal of 10 discs for each time point to estimate when Dpp^{HA} is not detected anymore. Representative graphs are shown in Fig. 3E-H. Both approaches allowed us to independently estimate when the first copy of the tandem could not be found in the dorsal region anymore. In both experiments, we found that Dpp^{HA} is almost completely gone 18 h after we shift larvae to 29ºC (Fig. 4B,H).

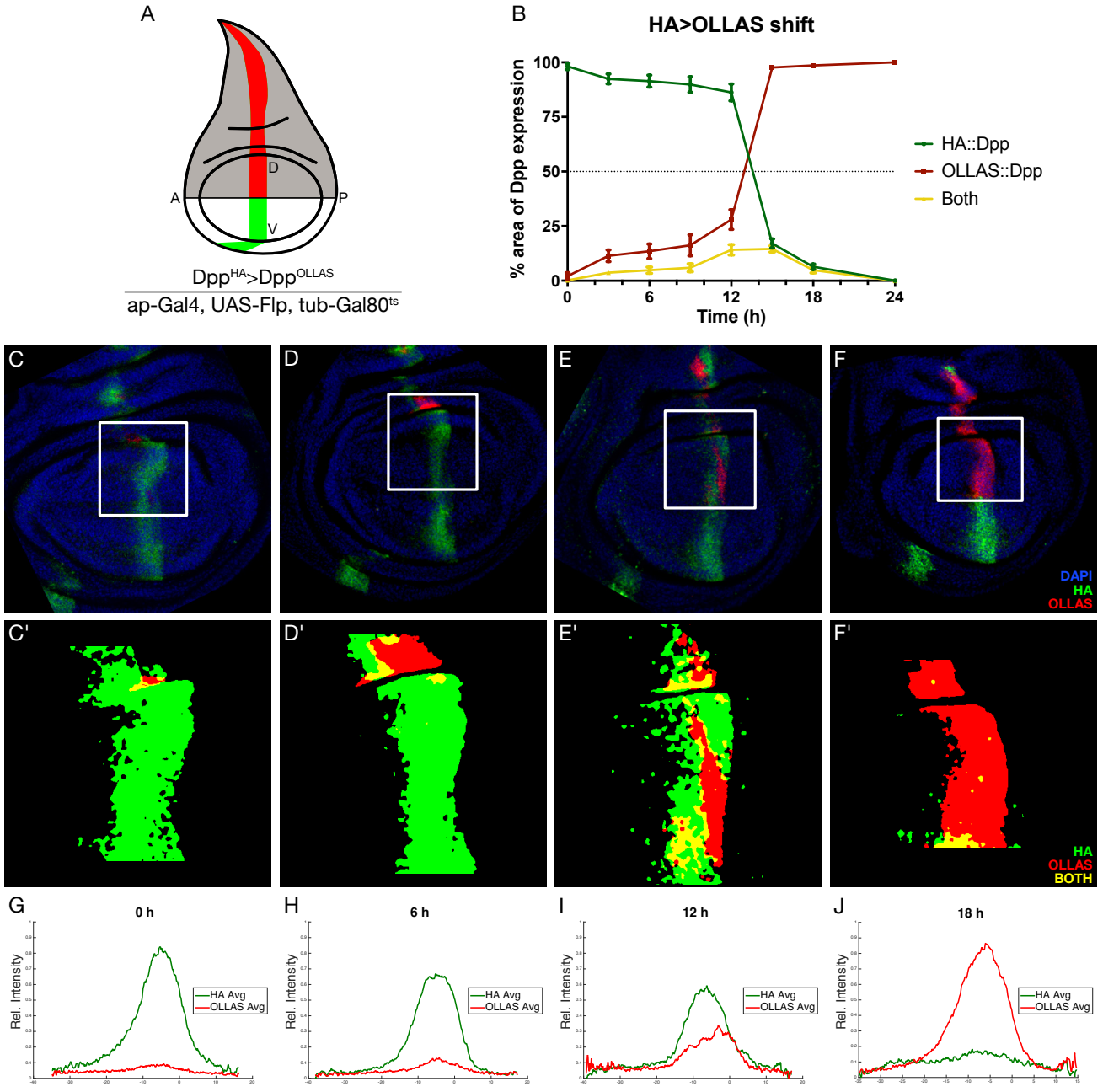

Figure 4. Efficiency of the Dpp tandem cleavage

A) Schematic of the experimental setup. Dpp cleavage only occurs in the dorsal compartment, while the ventral compartment remains as an internal control. B) Average area of cells expressing each of the two tandem copies over the timespan of 24 h, n = 10. C-F') Representative discs and the extracted Dpp-expressing areas used to measure the protein shift dissected without temperature shift (C-C'), 6 hrs on 29 ºC (D-D'), 12 hrs on 29 ºC (E-E') and 18 hrs on 29 ºC (F-F'). G-J) Intracellular measurements of Dpp^{HA} and Dpp^{OLLAS} at different time points, averaged from 10 discs.

Next, we estimated the extracellular fraction of Dpp^{HA} once the intracellular was undetectable. We could detect Dpp^{HA} staining in the dorsal compartment even 24 hrs after temperature shift (Fig 5F). That is, 6 h after Dpp^{HA} was no longer present in Dpp producing cells. That observation is incompatible with free diffusion, which would predict a fast turnover of the

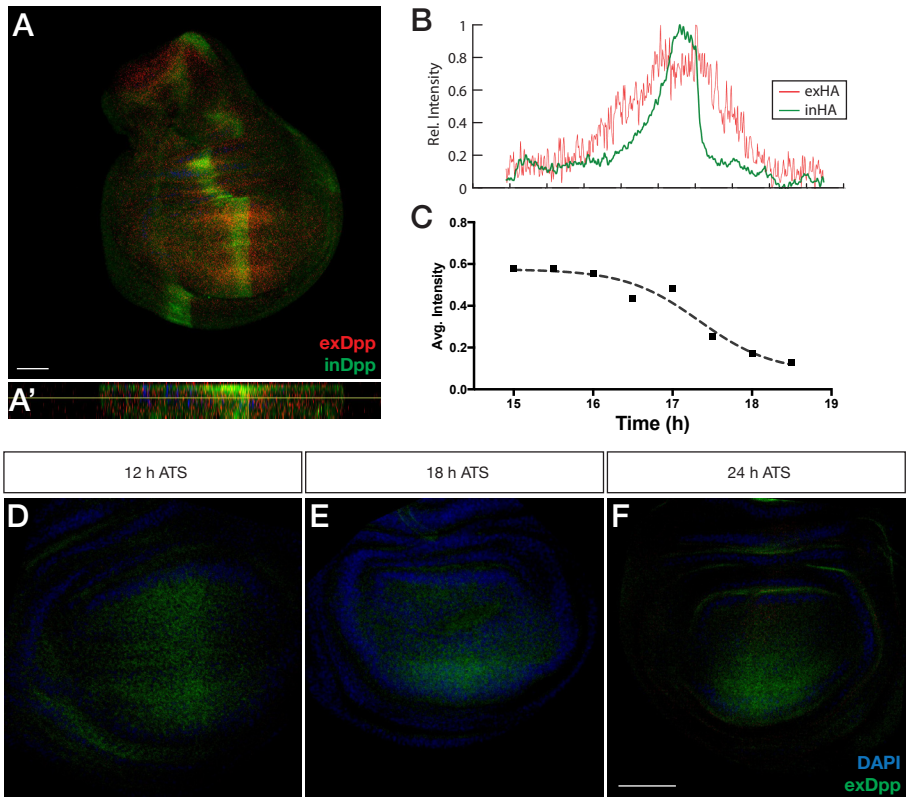

Figure 5. Decay of Dpp after cleavage

A) Expression of intracellular and extracellular Dpp^{HA} without Flp activity. A') Orthogonal view of the image stack from A. B) Intensity measuremnt of intracellular and extracellular Dpp^{HA} of the disc shown in A. C) Extracellular pool of Dpp^{HA} (exDpp) decay over the timespan of 5 hrs (15-19 hrs after Flp activation). Notice how Dpp starts decaying at 16 hrs after Dpp^{HA} excision from the genome, slowly dissapearing until 19 hrs, where around 20% of the exDpp is still present. D-F) Representative discs showing the Dpp^{HA} decay from 12 to 24 hrs after excision of Dpp^{HA}. Notice how after 24 hrs some Dpp is still detectable. Scalebar = 50 μM

protein. To further analyze and quantify Dpp degradation, we measured Dpp^{HA} presence at different time points, which allows us to estimate the degradation coefficient. Discs were stained from 15 to 24 h after temperature shift (representative discs depicted in Fig 5D-F), and the average Dpp^{HA} pool was calculated, using the ventral compartment as control. By observing the decay of Dpp^{HA} protein we estimate that the degradation of Dpp takes over 4 hrs, with some protein present up to 6 hrs after the intracellular pool is gone. Although these experiments are still ongoing, our preliminar results are more compatible with a hidered diffusion model, where Dpp takes around 6 hours to be degraded from the extracellular space.

Glypicans are main regulators of BMP signaling

Dpp can be hindered in various ways. While diffusing, it can bind to glypicans or receptors, thus reducing the movement of the morphogen. We wanted to test if altering the levels of glypicans, Dpp receptors or Pent, the expander molecule, affected Dpp signaling or diffusion. We used RNAi lines against Dally, Dlp, Tkv, Put or Pent, with the same genetic background used in the analysis of the Dpp gradient. The RNAi was expressed in the same region where we induced the tandem shift. Under these conditions, we assayed the effect of the gene downregulation on Dpp signaling. To do so, we measured the levels of intracellular Dpp and Brk to assess the effect

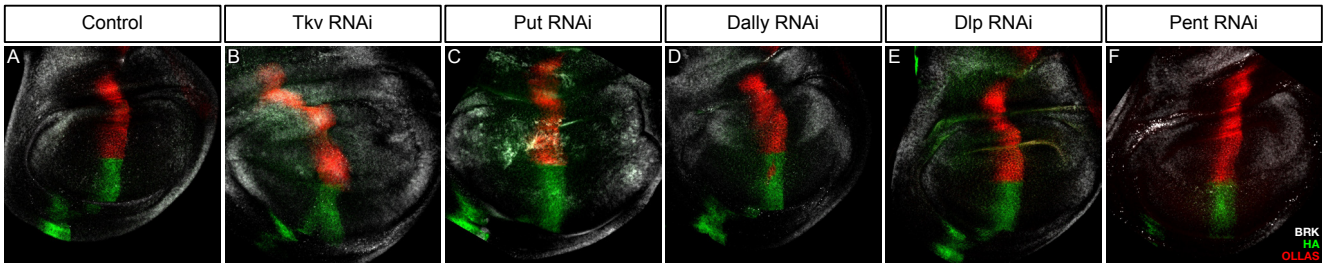

Figure 6. Role of glypicans, Dpp receptors and Pent in Dpp signaling

Staining against Dpp and Brk in wing discs expressing Flp recombinase and RNAi against Tkv (B), Put (C), Dally (D), Dlp (E) or Pent (F) in the dorsal compartment. A control disc without RNAi expression is shown for comparison in A. After expression of all RNAi, Brk was derepressed in the dorsal compartment. For Tkv, Put and Dally RNAi, the dorsal compartment presents delayed growth compared to the ventral compartment and the control disc. Dpp expression was not affected in any of the assays.

in Dpp signaling, and compared to the levels in the control ventral compartment.

We used various RNAi lines for each of the tested components, and selected the most efficient in terms of Brk derepression and lethality for the analysis. The most efficient RNAi lines and their effect in BMP signaling are shown in Fig 6. Consistent with previous research, we found that altering the levels of glypicans have a similar effect as the downregulation of Dpp receptors, as Brk was derepressed in the dorsal compartment. In addition, various RNAi showed growth impairment: the dorsal region of the pouch showed growth defects when Tkv or Dally were downregulated (Fig. 6B,D). These two RNAi are also the two that produced a higher BMP signal inhibition. Unfortunately, we cannot address if Tkv or Dally have a more prominent role in BMP signaling, or their RNAi are more efficient than those of Punt or Dlp.

Despite the different impact on BMP signaling, we could confirm that glypicans, receptors and Pent all are required for a proper Dpp signaling. It remains to be analyzed, whether they just affect signaling via ligand-receptor interaction or if they also alter the Dpp gradient. These experiments are ongoing.

Discussion

In this work we present a novel approach to study morphogen diffusion. For the first time, we managed to track endogenous Dpp diffusion and degradation, measuring extracellular levels and intensity decay after gene

knock-out. Since we measure endogenous morphogen levels, our model is more reliable than those based on Dpp overexpression.

Over two decades, several approaches have been followed to study Dpp diffusion. From eGFP-tagged Dpp (Kicheva et al., 2007; Rogulja and Irvine, 2005; Teleman and Cohen, 2000; Wartlick et al., 2011b) to photoconvertible fluorescent tags (Zhou et al., 2012), all models have been based in the expression of Dpp transgenes via $dpp^{BLK\text{-}Gal4}$. Our approach, in the other hand, measures a transgenic Dpp under the control of the endogenous locus, where the production and secretion of Dpp is much lower. We are confident that our design is much more suitable to quantify morphogen diffusion and degradation. Even considering that the signal-to-noise ratio of our approach is lower, our measurements are precise and were able to capture slight differences in the Dpp pool.

We found that the Dpp recycling is a very slow process, as the degradation of the Dpp^{HA} pool after stopping its production takes over 6 hrs (Fig 4F, 5E?). Our results do not support a model where Dpp diffuses freely but rather suggest hindered diffusion of the morphogen. Several authors have found data that pointed to restricted diffusion of morphogens (Schwank et al., 2011; Vuilleumier et al., 2010; Ben-Zvi et al., 2011). Most of these models propose that glypicans on the surface of the cells restrict the movement of morphogens, trapping them and creating a barrier for diffusion. Our data also suggests that morphogens are trapped after secretion and remain attached to the cell surface, where they can interact with the receptors. This morphogen pool is slowly degraded after internalization. Therefore by using the Dpp tandem, we could measure for the first time the extracellular Dpp population and obtained strong evidence suggesting that Dpp turnover does not fit with a free diffusion model, and points to a hindered diffusion of the morphogen, showing slow protein degradation and long retention at the cell membrane.

The mechanism by which Dpp diffuses is critical for the downstream signaling, as it determines the speed at which the receptors activate and the levels of signal transduction. Glypicans have been proposed to trap Dpp to expose it to the receptors, both controlling the intensity of the Dpp signaling and the shape of the gradient. In addition, the expander molecule Pentagone would regulate the levels of glypicans to enhance the range of the Dpp gradient and reduce the signaling where Dpp concentration is higher (Hamaratoglu et al., 2011; Norman et al., 2016; Vuilleumier et

al., 2010). We have observed that altering the levels of glypicans have a direct effect in Dpp signaling, as Brk is widely de-repressed. Growth is also impaired when Dally is downregulated (Fig. 6D), resembling to the lack of Tkv. These data are consistent with previous findings, where glypicans are required as co-receptors for proper BMP signaling (Jackson et al., 1997; Norman et al., 2016). It remains to be determined, if altering the levels of glypicans reduces the extracellular Dpp pool or it prevents the degradation of Dpp via receptor internalization.

In summary, we present a novel convertible transgene system to the study of the Dpp gradient and determine morphogen degradation and diffusion. With this model, one can analyze changes in the steady state while altering the levels of receptors, glypicans or other molecules related to BMP signaling such as Pentagone. We are convinced that the application of the Dpp tandem will allow us to deduce the mechanism by which the Dpp gradient is established and how it is regulated and scaled with tissue size.

Outlook

Our tool allows us to study the Dpp gradient in a level of detail that was not possible before and assess the molecular mechanisms underlying Dpp diffusion, as well as the key players in regulating the BMP gradient steady state. From here, we will focus on analyzing the interaction between the extracellular pool of Dpp and glypicans, receptors and Pentagone. Knowing the role of glypicans in trapping Dpp to activate BMP signaling, we hypothesize that manipulating the levels of glypicans will in turn affect the Dpp gradient. We plan to inhibit or overexpress both Drosophila glypicans, Dally and Dlp, then measure the shape of the Dpp gradient and the amount of morphogen remaining in the extracellular region. If glypicans are involved in maintaining the Dpp gradient, we should see how Dpp levels drop and the gradient widens when glypicans are downregulated. In the other hand, morphogen will concentrate mainly at the expressing region if glypicans are overexpressed, as most Dpp will be quickly trapped by the increased amount of binding partners.

Dpp receptors have also been proposed as mainly regulators of the Dpp gradient (Lecuit and Cohen, 1998; Schwank et al., 2011). Similar to glypicans, we expect that altering the levels of Dpp receptors will affect the morphogenetic gradient. Dpp binding to the receptors is known to be crucial for morphogen degradation. Therefore, reducing the levels of receptors

would potentially increase the amount of extracellular Dpp, and vice versa.

While glypicans and receptors may play a critical role in the establishment of the Dpp gradient, their levels have to be regulated to maintain a stable gradient. In addition, while tissue grows, the length of the gradient has to scale to the disc size. If, as we expect, glypicans regulate both BMP signaling and Dpp spreading, their levels have to be tightly regulated. Recently, it has been demonstrated that Pent fine-tune the levels of glypicans to regulate Dpp signal output. By internalizing glypicans, Pent adjusts the amount of morphogen trapped and presented to the Tkv and Put (Norman et al., 2016). We intend to analyze if Pent plays a key role in regulating the Dpp pool and gradient shape in addition to regulating BMP signaling. If so, altering the levels of Pent will expectedly affect the shape of the Dpp gradient, as well as the amount of morphogen presented at the cell surface. Decreasing Pent increases the levels of glypicans (Norman et al., 2016; Vuilleumier et al., 2010), which would expectedly increase Dpp trapping and restricts its diffusion. On the other hand, overexpressing Pent would trigger higher glypican internalization, extending the Dpp gradient and reducing the amount of Dpp at the cell surface.

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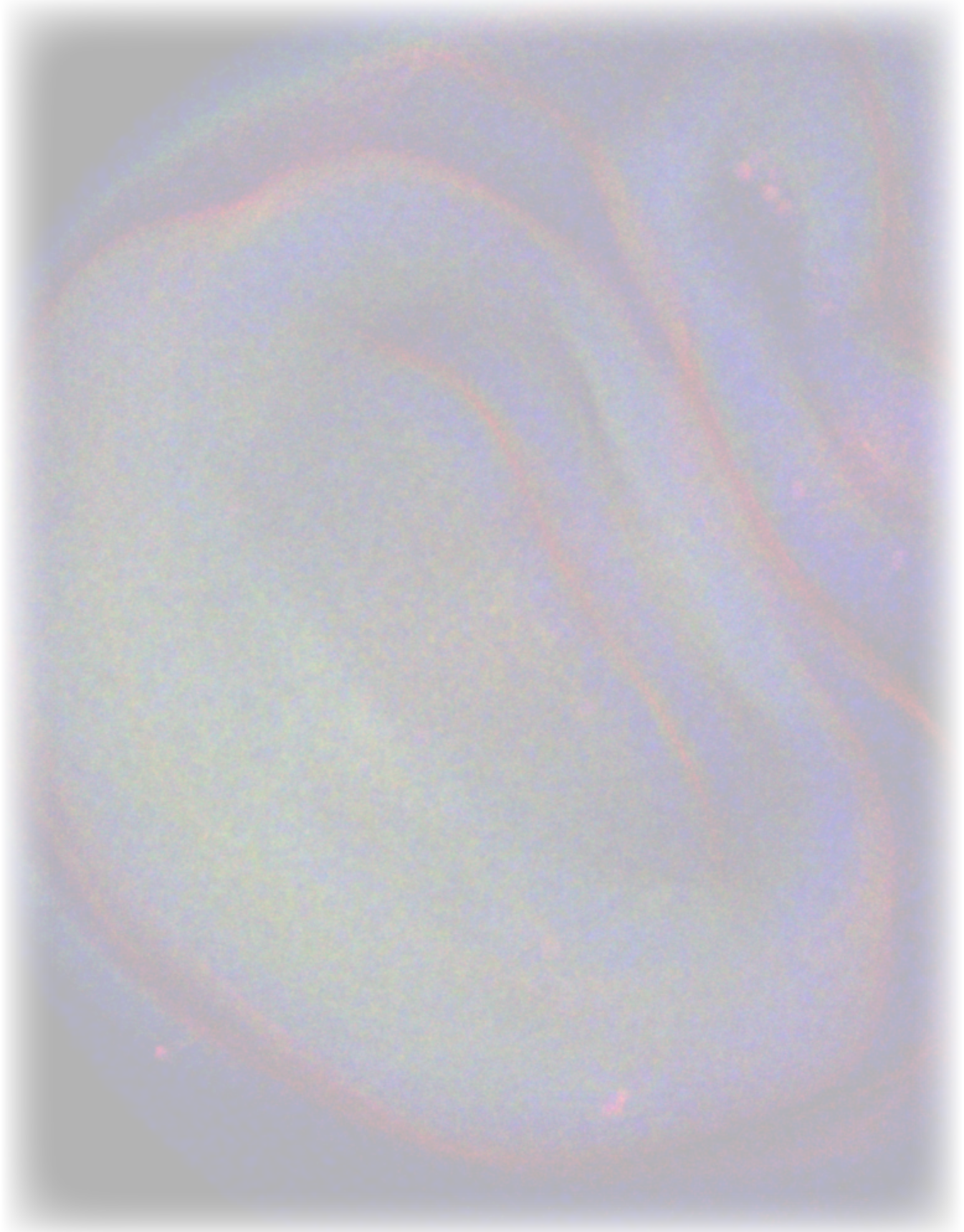
Chapter 5. Discussion and concluding remarks



In this thesis, I present two key advances in understanding the role of Dpp in development. First, I show how the graded signal from Dpp regulates growth in the wing imaginal disc. Second, I developed of a novel tandem Dpp transgene with which I have been able to show that Dpp diffuses slowly throughout the wing disc to generate the long range gradient.

In chapter 3, I presented a new *dpp* allele to produce conditional *dpp* KO. By taking advantage of the extensive toolbox of *Drosophila*, I generated compartmental and temporal LOF of *dpp* to study its effects in growth. We have found that Dpp emanating from the anteroposterior boundary is critical for growth, which led to the publication of an article in Elife.

In chapter 4, I use the *dpp* tandem transgene to follow the fate of the extracellular Dpp pool. I have been able to visualize for the first time the endogenous extracellular population of Dpp. I could therefore overcome the problems arising from studying Dpp diffusion with an overexpression Gal4>UAS model and study the mechanisms by which Dpp diffuses and generate a long-range extracellular gradient. We have found that the diffusion of Dpp is too slowly to be free diffusion, and I propose that Dpp diffuses steadily, hindered by extracellular proteins.

Understanding how a graded signal can drive homogenous growth

Finding how a graded morphogen signal regulates both patterning and growth has challenged scientists over decades. Some recent publications challenged the dogma that the Dpp gradient is required for growth. It was proposed that the Dpp gradient was only required for patterning but not for growth, and that only early Dpp signaling or a small, continuous source of Dpp in the tissue would suffice for growth (Akiyama and Gibson, 2015). Also recently, it was shown that eliminating the Dpp gradient by sequestering the morphogen to the source abolishes patterning while still allows tissue growth (Harmansa et al., 2015).

In this thesis, I provide evidence demonstrating that Dpp produced at the A/P boundary is indeed required not only for patterning, but also for growth. I show that abolishing Dpp expression at the AP boundary or the pouch leads to severe growth impairment, and a loss of patterning in the

wing disc (Sanchez Bosch et al., 2017). In agreement with my results, two other recent publications also find the medial expression of Dpp critical for tissue growth (Barrio and Milán, 2017; Matsuda and Affolter, 2017).

However, if the gradient of Dpp is required for growth, how is an uneven distribution of Dpp transformed into equal growth? This question has been investigated several times in the past but still remains a point of contention. The growth equalization model postulates that the main role of Dpp is to inhibit Brk, which inhibits growth in its expression domain. Lateral cells have been shown to divide faster than medial cells, either because the mechanical forces inherent to epithelial tissues (Aegerter-Wilmsen et al., 2007) or by signaling pathways delaying growth of medial cells (Schwank et al., 2011). Brk is in charge of limiting proliferation in the lateral regions, equalizing growth throughout the tissue. To block expression of Brk in the medial region of the disc, pouch cells need to be exposed to sufficient levels of Dpp to inhibit Brk and undergo normal growth. (Schwank et al., 2011; 2008). My findings confirm this hypothesis. Abolishing *dpp* expression resulted not only in patterning defects but also wing disc growth arrest. However, rescuing $dpp^{-/-}$ discs with homogeneous levels of Dpp throughout the pouch, was enough for the cells to proliferate. My results therefore point to Dpp having a dual role: 1) the graded morphogen expression triggers different dosage-dependent downstream signaling cascades, which are required for proper patterning. 2) a basal concentration threshold throughout the pouch just enough to repress Brk is required for growth.

Dpp hindered diffusion creates the morphogenetic gradient

In this thesis, I replaced the endogenous *dpp* by a tandem Dpp transgene to study the Dpp gradient. The first copy of this tandem is flanked by FRT sites, allowing switching of the gene product after FLP-mediate excision of the FRT-flanked fragment. After excision, one can track protein degradation by monitoring the disappearance of the transgene. Using this system I have been able to assess the endogenous levels of Dpp in the wing imaginal disc for the first time. Unlike former approaches which relied on overexpression, our system allows us to look at the expression and degradation of endogenous Dpp.

I found that the Dpp gradient, once established, is very stable and Dpp protein is present in the extracellular region for several hours after inducing the gene cleavage. Dpp remains trapped at the surface of the cells, most likely attached to glypicans. My data support the slow hindered diffusion model, where binding to glypicans reduces the propagation of Dpp and stabilizes the gradient.

It has previously been postulated that Dpp moves freely, based on the assumption that the population of free diffusible Dpp is too low to create a gradient via hindered diffusion (Zhou et al., 2012). The experiments of Zhou and collaborators, showed a population of fluorescently-tagged Dpp diffusing at a speed consistent with free diffusion. These observations led the authors to conclude that a freely-diffusing population establishes the long-range gradient, while the Dpp trapped at the surface of the disc cells belongs to a non-diffusive pool, involved in the receptor binding, signal transduction and subsequent internalization. If the observed free diffusible Dpp was an artifact of overexpressed Dpp causing an excess of morphogen that could not be retained by the glypicans due to saturation of their binding sites remains to be determined. By using our approach, I can track the second copy of the tandem after the protein production has started, so I can assess how much time is required to reach the steady-state gradient. If my findings determine that the protein gradient is rapidly generated after induction of the tandem shift, it will support the free diffusion model.

Maintenance of the steady state gradient

While Dpp diffusion is the first step to build up a gradient, it is the shape and the variability of the gradient that regulates developmental processes. Throughout the development of the wing disc, Dpp must maintain a concentration gradient to ensure the proper expression of patterning genes, and at the same time to the levels need to be above the threshold required to inhibit Brk in the pouch. Therefore, it is important to address 1) how the steady state gradient is regulated while the tissue grows, and 2) how it is scaled depending on the tissue size. Future experiments will go in that direction.

Various studies have suggested that a loss of glypicans destabilizes the Dpp gradient and disrupts BMP signaling (Akiyama et al., 2008; Jackson

et al., 1997; Norman et al., 2016). Glypicans trap Dpp ligand and keep it at the cell surface, where it will be able to bind to the receptors. Glypicans are not only involved in regulating Dpp/BMP signaling, but also play a role as co-receptors in Wnt, Hedgehog and fibroblast growth factor (FGF) signaling pathways (Baeg and Perrimon, 2000; Häcker et al., 2005; Lin and Perrimon, 1999). Their role as co-receptors is also conserved in mammals. They have been shown to bind Hh, FGF and Wnt ligands. Defects in glypicans impair the activation of several pathways and are linked to tumor progression (Filmus and Selleck, 2001; Sarrazin et al., 2011). Understanding glypican involvement in Dpp/BMP signaling is critical, as they could represent a general mechanism to regulate and coordinate several pathways involved in growth and patterning. I hypothesize that altering the levels of glypicans in the wing disc will dramatically reduce the amount of extracellular Dpp due to the inability to trap and concentrate the secreted morphogen. Consequently, the amount of morphogen presented to the Dpp receptors will be reduced, impairing BMP signaling. I have already confirmed that reducing the levels of the glypicans Dally and Dlp disrupts Dpp signaling. However, it remains to be proven whether reduced signaling is a result of a reduction in the extracellular Dpp levels or impairment in the ligand-receptor interaction.

The Dpp receptors Tkv and Put also play a role in the maintenance of the gradient. During the early studies of the Dpp gradient, it was found that Dpp internalization is triggered by binding to its receptors, as discs lacking Tkv accumulate extracellular Dpp and clones without Tkv are unable to internalize the ligand (Entchev et al., 2000; Teleman and Cohen, 2000). This internalization is critical to maintain the gradient and proper Dpp signaling once the steady state has been reached. In this context, an increase in the Dpp pool would result in more ligand binding to the receptors. On the other hand, regions with low Dpp signaling such as the cells at the lateral pouch receive a lower amount of Dpp. This translates in low Dpp signaling, but also a reduced internalization of morphogen. This mechanism will favor keeping a basal pool of ligand, enough to downregulate Brk and allow tissue growth. Modifying the levels of its receptors not only impairs the Dpp signal transduction, but also has an effect on the extracellular levels of Dpp. Increasing the levels of Tkv have shown to restrict the Dpp signaling to a narrow domain around the Dpp expressing region (Akiyama et al., 2008; Lecuit and Cohen, 1998). This insufficient Dpp expansion can be potentially caused by receptor-mediated endocytosis or by arres-

ting the Dpp diffusion (i.e. binding without internalization). It remains to be analyzed with our tandem system, whether altering the receptor levels affects the spreading of Dpp or only the degradation via receptor-mediated endocytosis. Analyzing the shape of the gradient or the size of the extracellular Dpp pool will allow us to know how the Dpp receptors shape the BMP signaling domain.

Finally, Pent might also be a critical player in determining the Dpp gradient. Its role as facilitator – also named expander – has been extensively studied (Ben-Zvi et al., 2011; Hamaratoglu et al., 2011; Vuilleumier et al., 2010). The expression of Pent is inhibited by Dpp signaling, therefore it is distributed in a mirror-imaged concentration gradient respect to the Dpp signaling gradient (Vuilleumier et al., 2010). By interacting with glypicans, Pent regulates the levels of Dally and Dlp to fine-tune not only BMP signaling, but also other signaling pathways, such as Wnt signaling, by reducing the amount of morphogens trapped at the cell surface (Norman et al., 2016). In this context, the role of Pent would be to avoid excessive trapping of Dpp at the source and the scaling of the gradient relative to organ size. The lateral region of the wing disc expresses Pent, which diffuses towards the center of the disc. As Pent binding results in internalization of glypicans, it reduces the trapping of Dpp, which can travel farther and extend the gradient to cover a broader surface. This mechanism ensures proper patterning of the tissue independently on its size, as growth would influence Pent expression (Hamaratoglu et al., 2011). Using our tandem system we can test, if increasing or decreasing the levels of Pent affects the extracellular pool of Dpp and the shape of the morphogenetic gradient.

The interaction between Dpp, glypicans, Dpp receptors and Pent represents a complex regulatory system, where all players seem to be equally involved in the establishment and maintenance of the Dpp gradient and signal transduction:

1. The glypicans oversee trapping Dpp molecules to concentrate them around cells and to restrict their diffusion.
2. Pent regulates the levels of glypicans to avoid excessive trapping around the A/P boundary.
3. Dpp receptors, on the other hand, are not involved in the establishment of the gradient, but in its maintenance, avoiding excessive extracellular Dpp by internalizing signaling molecules and reducing

the Dpp pool outside cells.

I am confident that the experiments laid out in this chapter using the Dpp tandem approach will help to fill the missing gaps and increase our understanding of how a morphogenetic gradient is established and maintained, so that the embryonic/primordial tissues develop into a proper adult structure.

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Appendix I — Plasmids and CRISPR reagents

List of primers

ID	Name	Created	Type
pSB1	pSKB-dpp(AAA)dppT2	01.2013	Miniprep
pSB2	pSKB-dpp-dppT2	02.2013	Miniprep
pSB3	pSKB-dpp(deGFP)dppT2	02.2013	Miniprep
pSB4	pSKB-dpp(3xdHA)dppT2	02.2013	Miniprep
pSB5	pSKB-dpp(3xdV5)dppT2	02.2013	Miniprep
pSB6	pSKB-dpp(dKate2)dppT2	02.2013	Miniprep
pSB7	pSKB-dpp(dmEOS2)dppT2	02.2013	Miniprep
pSB8	pSKB-dpp(dTRT)dppT2	02.2013	Miniprep
pSB9	pUAST-dpp(AAA)dppT2-attB Δwl	02.2013	Miniprep
pSB10	pUAST-dpp-dppT2-attB Δwl	02.2013	Miniprep
pSB11	pUAST-dpp(deGFP)dppT2-attB Δwl	02.2013	Miniprep
pSB12	pUAST-dpp(3xdHA)dppT2-attB Δwl	02.2013	Miniprep
pSB13	pSKB-dpp(6xdHA)dppT2	02.2013	Miniprep
pSB14	pSKB-dpp(2xdTRT)dppT2	02.2013	Miniprep
pSB15	pUAST>CD2-y+>dpp(AAA)dppT2-attB Δwl	02.2013	Miniprep
pSB16	pUAST>CD2-y+>dpp-dppT2-attB Δwl	02.2013	Midiprep, 1 ug/ul
pSB17	pUAST>CD2-y+>dpp(deGFP)dppT2-attB Δwl	02.2013	Miniprep
pSB18	pUAST>CD2-y+>dpp(3xdHA)dppT2-attB Δwl	02.2013	Midiprep, 1 ug/ul
pSB19	pUAST-dpp(3xdV5)dppT2-attB Δwl	03.2013	Miniprep
pSB20	pUAST-dpp(dKate2)dppT2-attB Δwl	03.2013	Miniprep
pSB21	pUAST-dpp(dmEOS2)dppT2-attB Δwl	03.2013	Miniprep
pSB22	pUAST-dpp(dTRT)dppT2-attB Δwl	03.2013	Miniprep
pSB23	pUAST>CD2-y+>dpp(3xdV5)dppT2-attB Δwl	03.2013	Miniprep
pSB24	pUAST>CD2-y+>dpp(dKate2)dppT2-attB Δwl	03.2013	Miniprep
pSB25	pUAST>CD2-y+>dpp(dmEOS2)dppT2-attB Δwl	03.2013	Miniprep
pSB26	pUAST>CD2-y+>dpp(dTRT)dppT2-attB Δwl	03.2013	Miniprep
pSB27	pUAST-dpp(deGFP)-attB Δwl	07.2013	Miniprep
pSB28	pLOT-HhGFP-attB	07.2013	Miniprep
pSB29	pUAST>CD2-y+>dpp(deGFP)-attB Δwl	07.2013	Midiprep, 600 ng/µl
pSB30	pLOT>CD2-y+>HhGFP-attB	07.2013	Midiprep, 150 ng/µl

pSB31	pSKB-dpp(3xdOLLAS)dppT2	08.2013	Miniprep
pSB32	pUAST-dpp(3xdOLLAS)dppT2-attB Δwl	08.2013	Miniprep
pSB33	pU6-BbsI-dpp5'chiRNA	10.2013	Midiprep, 1 ug/ul
pSB34	pU6-BbsI-dpp3'chiRNA	10.2013	Midiprep, 1 ug/ul
pSB35	pUAST>CD2-y+>dpp(3xdOLLAS)dppT2-attB Δwl	10.2013	Midiprep, 500 ng/ul
pSB36	pDPP-Dpp(eGFP)-attB	10.2013	Miniprep
pSB37	pDPP-Dpp(TRT)-attB	10.2013	Miniprep
pSB38	p669-Dpp(eGFP)	12.2013	Miniprep
pSB39	p669-Dpp(TRT)	12.2013	Miniprep
pSB40	RIV-F-Dpp(eGFP)-F-MCS3	12.2013	Miniprep
pSB41	RIV-F-Dpp(TRT)-F-MCS3	12.2013	Miniprep
pSB42	pGE-F-Dpp(eGFP)-F-MCS3	12.2013	Miniprep
pSB43	pGE-F-Dpp(TRT)-F-MCS3	12.2013	Miniprep
pSB44	RIV-attB1-F-MCS-F-MCS3	01.2014	Miniprep
pSB45	RIV-attB1-F-MCS-F-BglII	01.2014	Miniprep
pSB46	RIV-attB1-F-dpp(eGFP)dppT2-F-BglII	01.2014	Midiprep, 1 ug/ul
pSB47	RIV-attB1-F-dpp(TRT)dppT2-F-BglII	01.2014	Miniprep
pSB48	RIV-attB1-F-dpp(eGFP)dppT2-F-dpp(TRT)	02.2014	Midiprep, 1 ug/ul
pSB49	RIV-attB1-F-dpp(TRT)dppT2-F-dpp(eGFP)	02.2014	Midiprep, 1 ug/ul
pSB50	RIV-attB1-F-dpp(3xHA)dppT2-F-BglII	05.2014	Midiprep, 410 ng/ul
pSB51	pV5Q-LHV1-attB	07.2014	Midiprep, 1 ug/ul
pSB52	Brk-LHV1-attB	07.2014	Midiprep, 650 ng/ul
pSB53	pU6-BbsI-Tor_chiRNA	07.2014	Midiprep, 1 ug/ul
pSB54	RIV-attB1-F-dpp(NotI)dppT2-F-BglII	07.2014	Midiprep, 1 ug/ul
pSB55	RIV-attB1-F-dpp-dppT2-F-BglII	07.2014	Midiprep, 1 ug/ul
pSB56	RIV-attB1-F-dpp(3xHA)dppT2-F-dpp(3xOLLAS)dppT2	09.2014	Miniprep
pSB57	RIV-attB1-BP-3'SS-F-dpp(eGFP)dppT2-F-dpp(TRT)	10.2014	Midiprep, 1 ug/ul
pSB58	RIV-attB1-BP-3'SS-F-dpp(TRT)dppT2-F-dpp(eGFP)	10.2014	Midiprep, 1 ug/ul
pSB60	RIV-attB1-BP-3'SS-F-dpp(NotI)dppT2-F	11.2014	Midiprep, 1 ug/ul
pSB61	RIV-attB1-BP-3'SS-F-dpp-dppT2-F	11.2014	Midiprep, 1 ug/ul
pSB62	RIV-attB1-BP-3'SS-F-dpp(GFP)dppT2-F	11.2014	Midiprep, 1 ug/ul
pSB63	RIV-attB1-BP-3'SS-F-dpp(HA)dppT2-F	11.2014	Midiprep, 1 ug/ul
pSB64	pDsRed-G17-FRB-GFP	03.2016	Miniprep
pSB67	pDsRed-FRB-GFP	03.2016	Miniprep
pSB68	pDsRed-FRB-GFP-pygoNterHR2	08.2016	Miniprep
pSB69	pDsRed-pygoNterHR1-FRB-GFP-pygoNterHR2	09.2016	Midiprep, 1 ug/ul
pSB70	pU6-BbsI-pygoCter-chiRNA	11.2016	Miniprep

pSB71	pU6-BbsI-pygoNter-chiRNA	11.2016	Miniprep
pSB72	RIV-attB1-BP-3'SS-F-dpp-dppT2-F-dpp(GFP)	01.2016	Miniprep
pSB73	pGEM-FRB-GFP	03.2016	Miniprep
pSB74	pGEM-G17-FRB-GFP	03.2016	Miniprep
pSB75	pGEM-PygoCterHR1	08.2016	Miniprep
pSB76	pGEM-PygoCterHR2	08.2016	Miniprep
pSB77	pGEM-PygoNterHR1	08.2016	Miniprep
pSB78	pGEM-PygoNterHR2	08.2016	Miniprep

List of CRISPR reagents

ID	Name	Sequence	Description
nSB17	Dpp chiRNA 5' F	CTTCGACATGGGTTAAGCGCTGGT	Used to generate pSB33
nSB18	Dpp chiRNA 5' R	AAACACCAGCGCTTAACCCATGTC	Used to generate pSB33
nSB19	Dpp chiRNA 3' F	CTTCGACGCTGGTGCTCGACCGCG	Used to generate pSB34
nSB20	Dpp chiRNA 3' R	AAACCGCGGTCGAGCACCAGCGTC	Used to generate pSB34
nSB21	Dpp ssODN	TCCATTCCGGTCCACATCCGAACCCACATC CGAATCCTATCCGAAGCCACCTAACCC TTGCCGACCCTACGCCCCCAACTGAGA GAACTCAAAGGTTACCCCAGTTGGGGCAC TACGCGAGGTGGCCTCCATCAACGTGCCC GCCAACGCCAAGGCCATCATCGCCGAGCA GGGCCCGTCCA	ssODN to integrate attP into the dpp locus
nSB58	NotI-AarI-FRB F1	AAGCGGCCGCGGACATATGCACACCTGC GATCATCCTCTGGCATGAGATG	Used to generate pSB67
nSB59	BglII-SapI-GFP R1	AAAGATCTTTACTAGTGCTCTTCTTTGTA TAGTTCATCCATG	Used to generate pSB67
nSB75	pygo Nter HR1 F	AAAACACCTGCAAAATCGCTCGCTTCTTC GCACGCAA	Used to clone pygo HR1 into pSB68
nSB76	pygo Nter HR1 R	AAAACACCTGCAAAAGGATATATGGCGCCA TACCAAG	Used to clone pygo HR1 into pSB68
nSB77	pygo Nter HR2 F	AAAAGCTCTTCACAAACGATTGCCGGGTC CAGCG	Used to clone pygo HR2 into pSB67
nSB78	pygo Nter HR2 R	AAAAGCTCTTCAGACCGGTGGCTGCGGAG GTTG	Used to clone pygo HR2 into pSB67
nSB81	pygo Nter gRNA F	CTTCGCTGGACCCGGCAATCGATA	Used to generate pSB71
nSB82	pygo Nter gRNA R	AAACTATCGATTGCCGGGTCCAGC	Used to generate pSB71

Appendix II — List of abbreviations

A/P	Anteroposterior
AA	Anchor away
AEL	After egg laying
Arm	Armadillo
BAC	Bacterial artificial chromosome
BMP	Bone morphogenetic protein
Brk	Brinker
CDS	Coding sequence
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated protein 9
Dally	Division abnormally delayed
Dlp	Dally-like protein
Dpp	Decapentaplegic
DSRF	Drosophila serum response factor
En	Engrailed
FGF	Fibroblast growth factor
FKBP12	FK506 binding protein 12
Flp	Flippase
FRB	FKBP12 rapamycin binding
FRT	Flippase recognizion target
GFP	Green fluorescence protein
gRNA	Guide RNA
H3	Histone 3

HA	Hemagglutinin
Hh	Hedgehog
HINGS	Heat-inactivated goat serum
HS	Heat shock
Hth	Homeothorax
KO	Knock-out
LOF	Loss of function
Mad	Mother against Dpp
mEOS2	monomeric Eos fluorescent protein 2
mKATE2	Monomeric Katusha 2
OLLAS	E.coli OmpF Linker and mouse Langerin fusion Sequence
Omb	Optomotor blind
PCR	Polymerase chain reaction
Pent	Pentagone
Put	Punt
Pygo	Pygopus
qRT-PCR	Quantitative real-time PCR
RFP	Red fluorescent protein
Rn	Rotund
Rpl13a	Ribosomal protein L13A
Salm	Spalt major
spPCR	Splinkerette PCR
tagRFPt	tag red fluorescent protein
TGF-β	Transforming growth factor β

Tor	Target of rapamycin
Tkv	Thickveins
UAS	Upstream activation sequence
UTR	Untranslated region
Wg	Wingless
WM1	Wing medium 1
WT	Wildtype

Appendix III — Curriculum Vitae

Personal Information

Name	Pablo SÁNCHEZ BOSCH
Present address	Zürichholzstrasse 5, 8057, Zürich
Phone	+41(0)765226544
Email	pablo.sanchezbosch@uzh.ch
Date of Birth	25.08.1985
Place of Birth	Almería, Spain
Citizenship	Spanish

Academic Education

2013-present	PhD thesis in Molecular Biology, in the lab of Prof. Dr. Konrad Basler at the Institute of Molecular Life Sciences, University of Zurich, Switzerland. **Thesis:** The Dpp morphogen gradient regulates growth and patterning in the *Drosophila* wing imaginal disc
2010-2011	M.Sc in Genetics and Cell Biology, Faculty of Biology, Universidad Complutense de Madrid, Spain. Thesis completed in the lab of Dr. Ignacio Moreno de Alborán **Thesis:** *In vivo* function of the proto-oncogene c-Myc during terminal B cell differentiation
2003-2010	B.Sc + M.Sc in Biology. Genetics and Biotechnology specializations. Universidad Complutense de Madrid, Spain. One year abroad in Georg-August Universität Göttingen. Thesis completed in the lab of Prof. Dr. Ernst A. Wimmer. **Thesis:** Genetic stabilization and improvement of transgenic sexing strains of the Mediterranean fruit fly *Ceratitis Capitata*.

2003	High school (Matura), Instituto de Educación Secundaria Diego Velasco, Torrelodones, Madrid, Spain.

Presentations at international research conferences

2016	**Oral presentation:** The Allied Genetics Conference 2016, Orlando, FL, USA Tracking morphogens down: Uncovering the Dpp morphogen gradient
2015	**Poster presentation:** The American Drosophila Research Conference 2015, Chicago, IL, USA Anchoring proteins away: A fast and regulated method to deplete proteins in the cell.

Publications and manuscripts in preparation

Sanchez Bosch, P., Ziukaite, R., Alexandre, C., Basler, K., and Vincent, J.-P.B. (2017). Dpp controls growth and patterning in Drosophila wing precursors through distinct modes of action. Elife *6*, e22546.

Sanchez Bosch, P., Pepperl, J., Basler, K. Anchor-away - a fast, reliable and reversible technique to inhibit proteins in *Drosophila melanogaster*. *In preparation.*

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I want to specially thank George for all these things: a) scientific input; b) critical reading of the manuscripts and this thesis; c) being the pillar that keeps the lab standing and prevents that we run like headless chickens when there is a problem; d) wine and beer expertise, and finding the best cocktail bars in town (and teaching me how to pronounce "crow").

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Thanks also to our great assistants/managers for all the work that seamlessly kept the lab running. Esther for keeping our flies alive and our buffers ready; and Eliane for all the sequencing, the cloning we did together and the baking tips (and the tasty fruit jams).

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ner ever. I always looked forward to every Thursday afternoon coffee to discuss about soccer and snow sports. I loved these L floor balcony discussions and I hope you agree with me that we both passed our language exams with honors.

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Thanks to Nick for not only being the annoying winner on the game evenings but for all the fun anecdotes of his Greek parties, the continuous discussions about basketball, our "let's call this basketball" games, all the jokes and laughs and showing us his appreciation for *ze* Germans. Ah, and also for the scientific discussions (everybody keeps forgetting that you worked with flies for many years and that you are capable of giving good scientific input).

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